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It also has been observed that the clot forming from polycythemic blood is fragile and may dissolve or become fluid upon minimal agitation.<sup>12</sup>

It is the purpose of this paper to report the results of a one and one-half year study of blood coagulation in forty-five patients with polycythemia vera, twenty-eight patients with leucemia and sixteen patients with other diseases. During the course of this study, two new tests of blood coagulation were developed and carefully standardized upon sixty-four normal subjects. These tests are:

1. Clot retraction rate. A quantitative measure of clot retraction obtained by electric resistance measurements.

2. Heparin clotting time. A modification and simplification of the heparin tolerance test, which measures the effect of added heparin upon the clotting time of blood.

#### METHODS

All blood coagulation tests were performed in a water bath regulated at 37° C. upon venous blood carefully drawn to avoid tissue juice contamination. A dry syringe and 20 gauge needle were used. The first and last cubic centimeters of blood in the syringe were not used for these tests. Blood counts were performed upon capillary blood.

*Clot Retraction Rate.*—A method\* was developed and described by Rosenthal and Tobias<sup>13</sup> by which a quantitative measure of clot retraction, as well as other information, may be obtained by the measurement of the electric resistance of freshly drawn blood, as it clots. Fig. 1 shows a typical electric resistance curve with explanations and illustrations of the changes during coagulation.

Since the increase in electric resistance is caused by retraction of the clot around the electrodes, the slope of the rising portion of the resistance curve is comparable to the "clot retraction rate." The resistance increase was usually linear and most rapid during the forty minutes of measurement. This period also was subject to the least amount of and variation such as result from the edge of the electrode cutting through the remaining clot. The clot retraction rate is calculated in the following manner:

$$\text{Clot retraction rate} \quad = \quad \frac{\text{Specific resistance at 35 min.} - \text{Specific resistance at 15 min.}}{35 \text{ min.} - 15 \text{ min.}}$$

in ohm-centimeters per minute

Normal range: 4 to 10 ohm-cm. per minute; average value: 7.1 ohm-cm. per minute.

*Heparin Clotting Time or Heparin Prolonged Clotting Time.*—This test measures the effect upon the clotting time of the addition of .004 mg. of heparin to 1 c.c. of venous blood. The use of this amount of heparin was arrived at by an extensive study upon the effects of various amounts of heparin upon the clotting time of blood.

Pyrax test tubes, 100 mm. by 13 mm. in dimensions, were marked with a scratch at a level coincident with the bottom part of the meniscus of 1.1 c.c. of fluid. Heparin was diluted to provide .004 mg. per 0.1 c.c. of isotonic saline; 0.1 c.c. of this heparin solution was placed in a dry test tube prepared as described. The clotting time was then determined after placing freshly drawn blood in the tube up to the scratch mark, thereby adding 1 c.c. of blood. The blood and heparin were thoroughly mixed by inverting the tube twice. A stopper was inserted and the tube was examined with minimal tilting and agitation for the formation

\*The method of electric resistance measurement was revised by Dr. C. W. Tobias, assisted by Mr. L. Lipetz. Measurements, since June, 1945, have been obtained by comparing the potential across the electrodes to a standard potential. A constant current is used, and the resultant potential across the electrodes is directly related to the electric resistance. Measurements are recorded automatically by a 6 channel Speedomax (Leeds and Northrup) every

<sup>13</sup>Heparin made by Abbott Laboratories was used in this study. Most commercial preparations, which may be used in this test, consist of the purified sodium salt of heparin in solution, 1 c.c. containing 10 mg. of heparin.

of a firm clot at one-minute intervals beginning at twelve minutes. The end point or clotting time occurred when the tube could be completely inverted without any fluid breaking from the clot. Careful attention to three factors in the technique eliminated much error and variation. 1. Precise measurement of the volumes of heparin solution and blood. The use of a

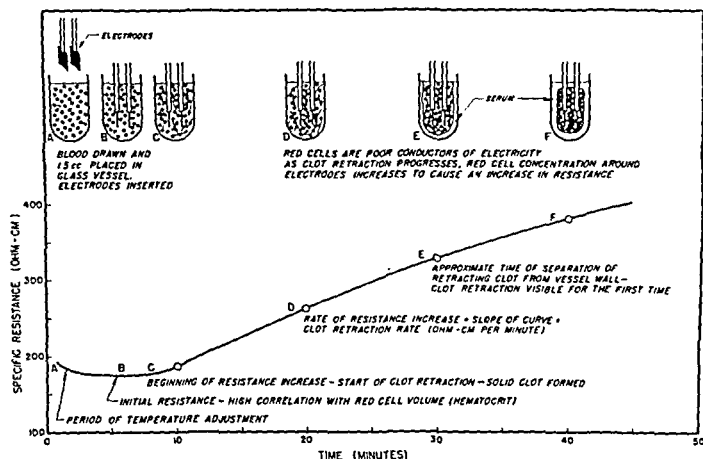


Fig. 1.—Typical electric resistance curve with explanations and illustrations of the changes during coagulation.

pipette for the measure of blood was not necessary if the relation between the scratch mark and meniscus of the blood was consistent. 2. Thorough mixing of the heparin with the blood. 3. Minimal agitation of the blood in the determination of the clotting time. In the course of this study it was possible to obtain good end points for clotting times as long as five hours. Duplicate measurements upon aliquot samples of blood showed an average variation of less than 5 per cent.

Normal range: 20 to 35 minutes. Average value: 27.5 minutes.

**Clotting Time (Lee-White).**—This is a variation of the Lee-White clotting time. One cubic centimeter of blood was placed in a dry, Pyrex tube, 100 mm. by 13 mm. in dimensions. The tube was examined for clot formation by minimal tilting every half minute. Normal range: 5 to 10.5 minutes.

**Toluidine Blue Titration Test.**—This test measured the effect of a series of concentrations of toluidine blue on the clotting time of recalcified oxalated plasma. Venous blood was mixed with 0.1 molar sodium oxalate, nine parts to one, and centrifuged for fifteen minutes at medium speed in a small clinical centrifuge. One-tenth cubic centimeter of plasma was added to each of six increasing amounts of toluidine blue\* in 0.05 c.c. of isotonic saline. One-tenth cubic centimeter of .025 molar calcium chloride solution was added to each tube which was then examined every fifteen seconds for clot formation. A control tube containing saline without toluidine blue was also measured.

**Clot Retraction by Inspection.**—The amount of clot retraction was estimated by external inspection of the clots formed in the electric resistance, heparin clotting, and Lee-White clotting tubes at four and twenty-four hours.

\*Toluidine blue O, made by National Aniline Division, Allied Chemical and Dye Corp., New York, N. Y. Total dye content was 69 per cent.



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†Heparin made by Lederle Laboratories was used in this study. Most commercial preparations, which may be used in this test, consist of the purified sodium salt of heparin in 5 ml. or 1 c.c. containing 10 mg. of heparin.

forty-one patients with polycythemia vera had values above the normal range, indicative of increased clot retraction. The distributions for the other groups are apparent.

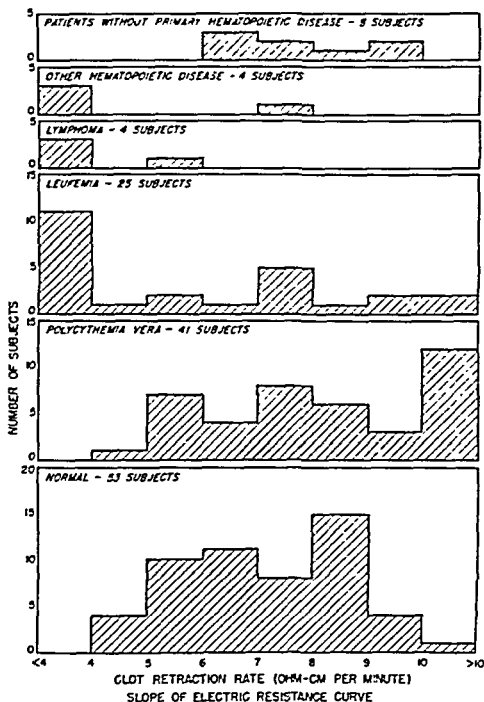


Fig. 2.—Distribution of results of the clot retraction rate for the various groups of subjects.

The relative effect of various factors involved in clot retraction upon the clot retraction rate was indicated by further analysis of the data and examination of the pattern of the electric resistance curves. The platelet count appeared to be the most important factor, showing a high correlation coefficient with the clot retraction rate of  $+0.83$ . The hematocrit, as indicated by the initial blood resistance, had only a negligible effect upon the clot retraction rate. The presence of an abnormally rapid red cell sedimentation rate showed its effect upon the pattern of the electric resistance curves as illustrated and explained in Fig. 3, A and B. This unusual pattern, called the "atypical electric resistance curve," usually occurred in the blood of leucemic patients with low hematocrits and rapid red cell sedimentation rates. The results indicate, how-

ever, that the clot retraction rates computed from these curves are not significantly altered by the red cell sedimentation. Fig. 3, C and D, gives examples of resistance curves giving low and high clot retraction rates respectively. Twelve duplicate measurements on aliquot samples of blood showed an average variation in clot retraction rate of 10 per cent.

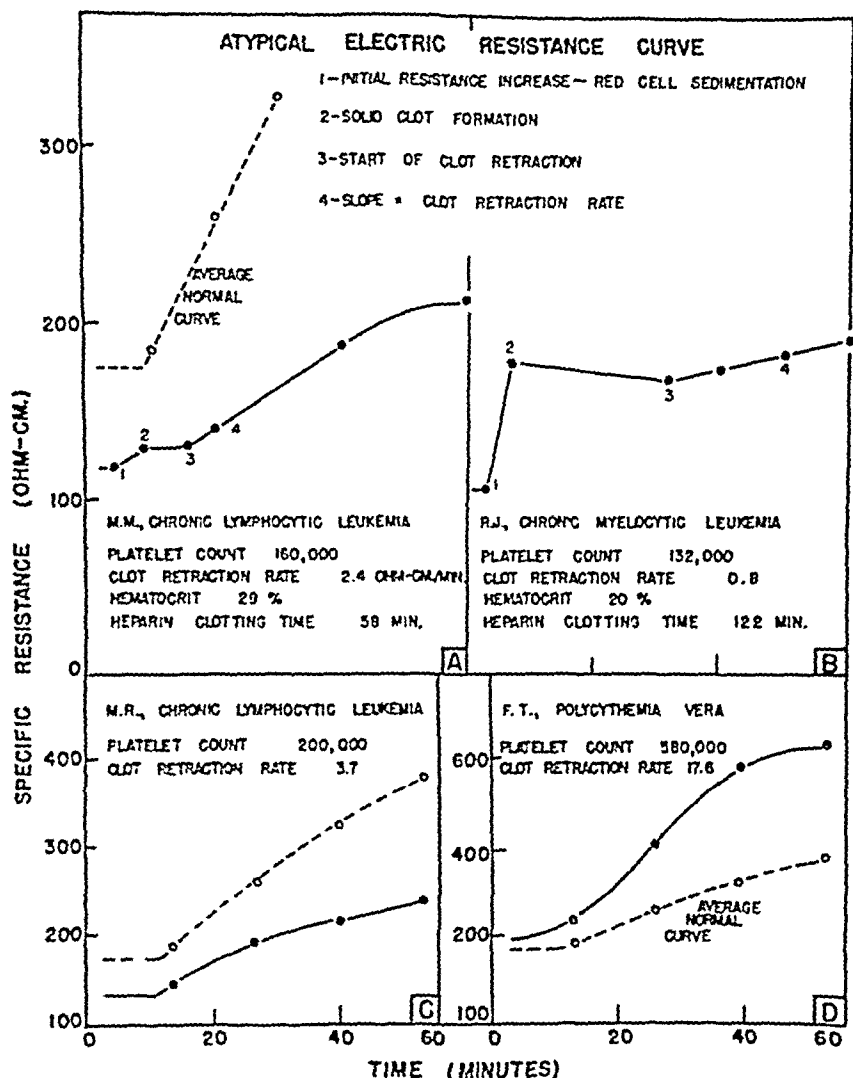


Fig. 3.—A and B, Examples and explanation of the atypical electric resistance curve. C and D, Examples of typical electric resistance curves which gave decreased and increased clot retraction rates respectively.

**Heparin Clotting Time.**—The results of this test are shown in Fig. 4. Thirty-three normal subjects showed a normal distribution of values. Eleven of twenty-one leucemic patients showed a definite prolongation of varying degree up to 155 min., as compared with the upper limit of normal of 35 minutes.

In polycythemia vera, twenty-four patients had normal values, three values were rapid and eight prolonged. The effect of an elevated hematocrit upon these results merits special consideration. Since the standard amount of heparin is added to a constant volume of blood, samples with higher hematocrits will have a correspondingly higher heparin concentration in the plasma. As red cells do not appear to play any active part in the clotting process, the higher heparin

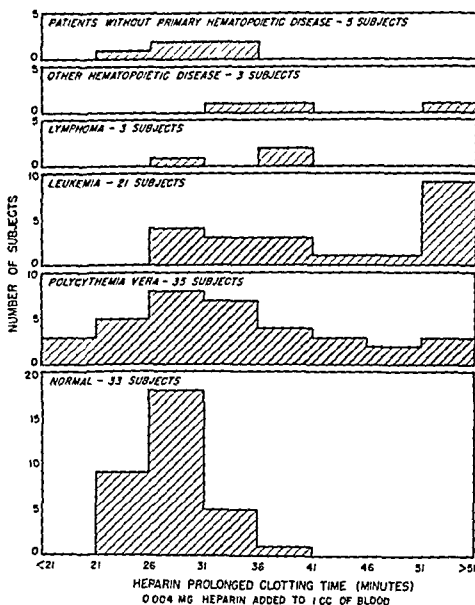


Fig. 4—Distribution of results of the heparin clotting time (or heparin prolonged clotting time) for the various groups of subjects.

concentration in the plasma could conceivably increase the heparin clotting time. This was found to be true for one patient who had hematocrits of 71, 63, 58, and 49 per cent at the times of study. Correction of the heparin clotting times for hematocrit caused a shift of five patients in the normal range to the rapid range.

In the other groups, patients with lymphomas and other hematologic disease tended to show prolonged values while all patients without any primary hematopoietic disease were in the normal range.

*Clotting Time (Lee-White).*—As shown in Fig. 5, all the patients showed normal values for this test.

ever, that the clot retraction rates computed from these curves are not significantly altered by the red cell sedimentation. Fig. 3, *C* and *D*, gives examples of resistance curves giving low and high clot retraction rates respectively. Twelve duplicate measurements on aliquot samples of blood showed an average variation in clot retraction rate of 10 per cent.

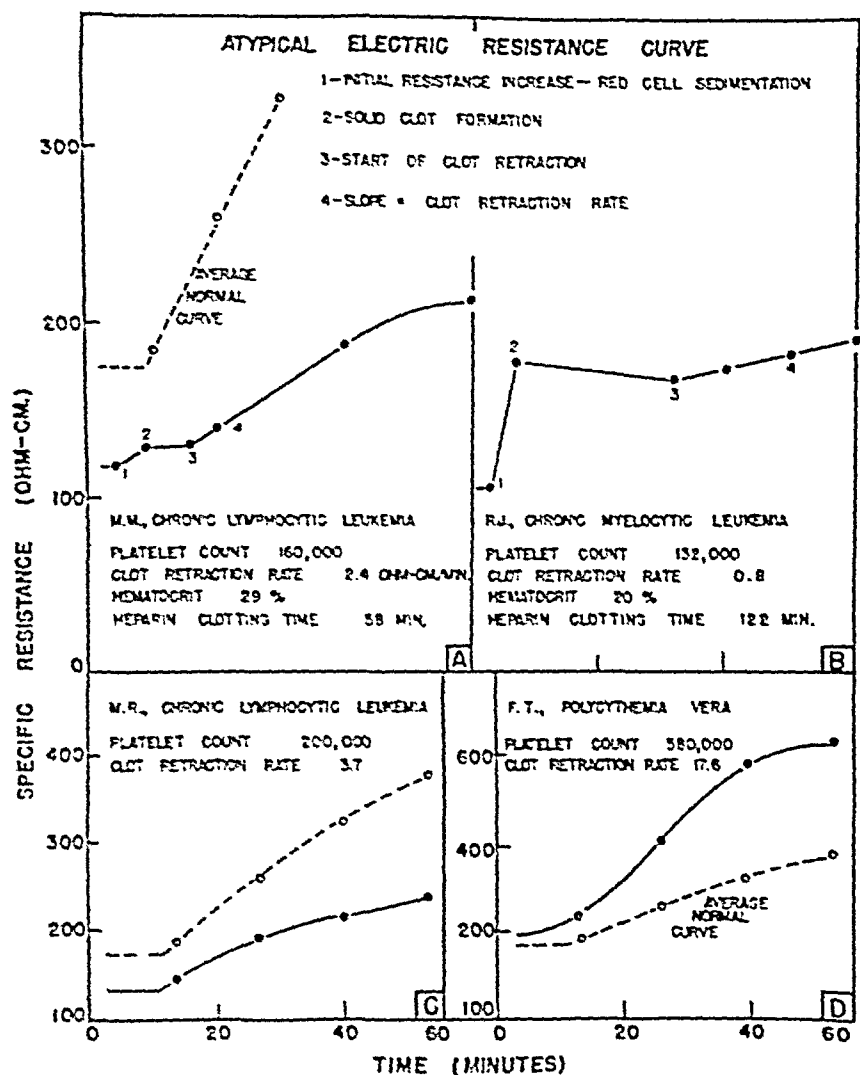


FIG. 3.—*A* and *B*, Examples and explanation of the atypical electric resistance curve. *C* and *D*, Examples of typical electric resistance curves which gave decreased and increased clot retraction rates respectively.

**Hypofibrin Clotting Time.**—The results of this test are shown in Fig. 4. Thirty-three normal subjects showed a normal distribution of values. Eleven of twenty-six leucemic patients showed a definite prolongation of varying degree up to 155 min., as compared with the upper limit of normal of 35 minutes.

*Prothrombin Time.*—This test gave normal results in nine patients with polycythemia vera, four with chronic leucemia, one with secondary polycythemia, and two with other diseases.

*Relation of Coagulation, Hematologic and Clinical Findings.*—

*Leucemia:* Patients with leucemia were divided into two groups, those with normal heparin clotting times below 40 min and those with values above 9 minutes. This revealed definite relations among certain coagulation tests, clinical evidence of bleeding, and hematologic status and course as shown in Table II.

In Group 1, where the heparin clotting times ranged from 26 to 40 min., no patient had any clinical evidence of bleeding except for one case in which vaginal bleeding was discovered to be caused locally by a uterine neoplasm. The clot retraction rates were normal except for a slight increase in one case. Platelet counts were all above 200,000.

In contrast, Group 2 showed heparin clotting times ranging from 44 to 155 minutes. Eight of the eleven patients in this group showed some clinical evidence of bleeding, the severity of which appeared related to the degree of prolongation of the heparin clotting time. The bleeding usually occurred from the nose, mouth, and gums and followed such procedure as a dental puncture. Purpura of the skin occurred in four patients. The bleeding could be graded as severe in only one subject. Eight of these patients showed impaired clot retraction with clot retraction rates below the lower normal limit of 4 ohm-cm. per minute. Eight patients had platelet counts below 200,000. The bleeding time was prolonged in only two of six cases with bleeding. In these six cases, the clot retraction rate was below normal and the heparin clotting time prolonged. The tourniquet test was negative in four patients with bleeding.

Hematologic findings differed in the two groups. Red cell counts and hematocrits were consistently higher in Group 1. White cell counts were similar.

A striking difference in the course of the disease was found between the two groups. Follow-up as of January, 1949, revealed that one patient in Group 1 and five patients in Group 2 had died.

*Polycythemia Vera:* In this series of thirty-nine patients, untreated except for a few phlebotomies, thirteen had histories of either thrombosis or hemorrhage. At the time of this study, no patient showed active evidence of these complications. Fourteen or 36 per cent of these patients had an elevation in platelet count above 400,000; while twelve of thirty-six or 33 per cent had an increase in clot retraction rate. Table III presents the coagulation and hematologic data for these twelve patients whose clot retraction rates ranged from 10.2 to 17.6 ohm-cm. per minute. Nine of these patients had platelet counts above 400,000. The heparin clotting time appeared to be generally in the normal range. After correction for hematocrit, eight of thirty-five untreated patients with polycythemia had rapid heparin clotting times. The bleeding time was normal in five patients. Hematologically, the untreated patients generally had elevated red and white cell counts and elevated hematocrits.

TABLE II. COAGULATION AND OTHER DATA FOR LEUKEMIC PATIENTS

QUANTIT	SEX	TYPE OF DYSPLASIA	DATE EX-AMINED (MO.-YR.)	HEPARIN CLOTTING TIME (MIN.)	CLINICAL EVIDENCE OF BLEEDING	CLOT RETRACTION RATE (CM. CM. PER MIN.)	CLOTTING TIME (LETT- WHITE) (MIN.)	PLATELET COUNT ( $\times 10^3$ )	BLEEDING TIME (MIN.)	RED CELL COUNT ( $\times 10^6$ )	HEMATO CRIT (%)	WHITE CELL COUNT	STATUS IN 19
<i>Group 1. Heparin Clotting Time Below 10 Min.</i>													
G. S.	F	C.M.L.	6-48	26	0	10.2	8	300	8	4.70	14	17,300	L
E. W.	F	C.M.L.	4-48	27	0	8.6	6	520	2 1/2	4.00	35	41,500	D 6-48
V. C.	M	C.L.L.	4-48	28	0	-	9	290	-	5.74	56	60,000	L
R. S.	F	C.L.L.	10-48	29	0	12.3	6 1/2	360	-	3.62	40	69,000	L
G. H.	M	C.M.L.	10-48	32	0	7.7	8 1/2	280	-	4.28	43	60,000	L
A. J.	M	C.L.L.	12-48	34	0	9.0	7 1/2	220	-	4.60	45	27,800	L
G. H.	M	C.L.L.	10-48	35	0	5.4	9	900	-	4.81	47	51,800	L
W. H.	M	C.L.L.	9-48	38	0	7.0	8 1/2	210	-	4.16	39	25,300	L
L. J.	F	C.M.L.	10-48	40	0	7.5	8 1/2	200	-	3.80	41	34,000	L
						9.1	6 1/2	360	-	3.50	36	125,500	L
<i>Group 2. Heparin Clotting Time Over 10 Min.</i>													
L. W.	M	A.L.	4-48	11	+	3.6	6	300	2	3.12	34	20,500	D 3-48
L. R.	F	C.M.L.	10-48	47	++	1.9†	6	230	3 1/2	2.08	20	51,000	D 11-48
H. W.	M	C.L.L.	4-48	53	0	5.1	8	120	5	4.46	43	21,900	L
T. P.	M	C.L.L.	6-48	58	0	6.8	6	300	-	4.10	41	16,800	L
P. R.	M	C.L.L.	9-48	58	0	2.44	8	160	3	2.90	29	10,300	L
C. S.	M	C.L.L.	5-48	62	+	.6	5 1/2	180	-	3.80	32	37,000	D 8-48
R. J.	F	C.L.L.	6-48	118	+	4.7	9 1/2	160	-	3.74	38	29,750	L
J. C.	F	C.M.L.	9-48	122	++	.8†	9 1/2	130	20	2.10	20	7,950	L
R. E.	M	C.L.L.	4-48	130	++	1.6	7	180	5	3.40	36	8,100	D 7-48
J. H.	M	C.M.L.	4-48	151	+++	.5†	4	110	11	1.73	20	90,000	D 5-48
				155	++	.1†	7	160	8	2.80	27	30,000	L

A.L., Acute leukemia.

C.M.L., Chronic myelocytic leukemia.

C.L.L., Chronic lymphocytic leukemia.

+, Slight bleeding; ++, moderate bleeding; +++, marked bleeding.

D, Dead; L, living.

\*Vaginal bleeding, subsequently discovered to be caused by uterine carcinoma.

†Atypical electric resistance curve.

TABLE III. COAGULATION AND HEMATOLOGIC DATA FOR POLYCYTHEMIA VERA PATIENTS WITH INCREASED CLOT RETRACTION RATES

PATIENT	SEX	DATE (MO.)	CLOT RE- TRACTION RATE (OHM-CM.)	PLATELET COUNT	HEPARIN CLOTTING TIME	CLOT- TING TIME	RED CELL COUNT ( $\times 10^9$ )	HEMATO- CRIT	WHITE CELL COUNT
A. F.	F	10-							
H. M.	M	9-48	19.3	200,000	29	9	7.34	66	16,600
L. G.	M	4-48	10.5	600,000	26	7	8.16	69	14,200
A. P.	M	10-47	11.1	470,000	-	8	7.70	58	21,800
H. K.	M	4-48	12.3	350,000	31	6	5.54	51.5	13,500
T. F.	M	6-48	13.2	400,000	25	6	5.12	52	12,500
E. W.	F	10-48	13.9	480,000	25	11	6.84	60	39,000
E. B.	M	11-48	13.9	580,000	20	8	8.30	61	33,000
G. T.	M	6-48	15.0	376,000	17	5	6.16	65	12,000
E. K.	F	10-48	15.1	490,000	43	10	7.45	62.5	10,300
C. R.	M	10-48	15.7	540,000	24	9	6.88	64	35,500
F. T.	M	11-48	17.6	580,000	24	8	6.42	56	13,750

In the clot retraction studies, inspection of the clots frequently showed little or no evidence of clot retraction; while, at the same time, the electric resistance curves indicated abnormally rapid retraction. When the electrodes were removed after twenty-four hours, a small, firm clot was often found well retracted between the electrodes. This was surrounded by red cells suspended in serum which composed most of the volume of the sample. This tended to occur chiefly in patients with both elevated hematocrits and platelet counts. These patients also gave poor end points of clotting time and sometimes a solid clot either never formed or rapidly dissolved.

Four patients in hematologic remission had normal clot retraction rates. Platelet counts were normal in three patients, slightly depressed below 200,000 in one. Heparin clotting time was normal in two and prolonged in two. Of the two patients in the leucemic phase of polycythemia, one had a depressed platelet count and low clot retraction rate, while the other had normal values for these tests and for the heparin clotting time.

*Other Diseases:* Because the number of patients in this category is small and the types of diseases varied, only some general observations will be made. Patients with lymphomas and other diseases related to the hematopoietic system generally showed some bone marrow depression as evidenced by anemia and slight thrombocytopenia. None had any sign of hemorrhage. These patients tended to have prolonged heparin clotting times and lowered clot retraction rates. Patients without hematologic disease and without evidence of bone marrow depression had normal platelet counts, clot retraction rates, and heparin clotting times. The two patients with polycythemia secondary to congenital cardiac abnormalities had normal values for these tests.

#### DISCUSSION

The results of this study have revealed certain new findings relative to blood coagulation in leukemia and polycythemia vera.

One-half of the patients with either chronic myelocytic or chronic lymphocytic leukemia showed a blood coagulation defect characterized by slight



method presented in this report has been devised to eliminate these difficulties. The test can readily be adapted to routine clinical use for the detection of either a rapid or slow clotting process. In addition to its diagnostic value, it can serve as a valuable therapeutic guide.

#### SUMMARY

1. Two methods for the measurement of blood coagulation are presented: the heparin clotting time and the clot retraction rate. The heparin clotting time measures the clotting sensitivity of the blood to added heparin. The clot retraction rate is a quantitative measure of clot retraction obtained by electric resistance measurements. Both tests were well standardized on a large number of normal subjects. Clearly defined upper and lower limits of normal were established.

2. In chronic leucemia, both myelocytic and lymphocytic, one-half of the patients showed a previously unreported coagulation defect characterized by a prolongation of the heparin clotting time, decreased clot retraction rate, and a slight thrombocytopenia between 110,000 and 180,000. The severity of this defect was closely related to the degree of hemorrhagic symptoms. No free or neutralizable heparin was detected by a toluidine blue titration test in the blood of two patients with marked hemorrhagic symptoms and the foregoing clotting defect. Leucemic patients without this clotting defect and thus with normal values for the coagulation tests showed no evidence of bleeding. These patients were in better condition, both hematologically and clinically, and had a better prognosis than patients with the coagulation defect.

3. Of the untreated patients with polycythemia vera and elevated hematocrits, 33 per cent showed increased clot retraction rates and platelet counts elevated above 400,000. A similar percentage of these patients had histories of either thrombosis or hemorrhage. An explanation is given for the simultaneous presence of an increased clot retraction rate, external appearance of poor clot retraction, and the formation of fragile, readily dissolving clots in many of the patients. These studies indicate that the elevation in platelet count is the most important single factor in the occurrence of both hemorrhage and thrombosis in polycythemia. Therapy should aim toward the rapid reduction of an increased number of platelets.

4. The results of this study provide indications of the relative clinical value of the various coagulation tests. The inadequacies and limitations of the platelet count, bleeding time, tourniquet test, and clot retraction estimated by inspection are shown. The extremely limited value of the widely used Lee-White clotting time cannot be overemphasized. The clot retraction rate provides a useful, quantitative measure of clot retraction but it requires special equipment for its performance. The heparin clotting time appears to be the most valuable single test or index of coagulation. Readily adaptable to both routine clinical and experimental use, it enables the detection of either abnormally increased or decreased clotting ability.

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## STUDIES ON THROMBOCYTOPEN

### I. A RELIABLE TEST FOR THIS PRINCIPLE IN ORGAN HOMOGENATES AND IN URINE

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IN 1933 Torrioli and Puddu<sup>29</sup> discovered that an extract prepared from the spleen of a patient with idiopathic thrombocytopenic purpura contained an agent which injured the megakaryocytes in bone marrow cultures. The same principle was then found to be present not only in normal spleens, but in other normal organs as well (liver, lung, heart, lymph nodes, and omentum), although in apparently lesser concentrations. When a protein-free aqueous extract from a normal spleen was injected intravenously into rabbits, a considerable reduction of the platelets in the circulating blood became noticeable. Blood of the splenic vein contained more of this agent in comparison with blood of the splenic artery. Based on these findings, the hypothesis was advanced that thrombocytopenic purpura may be caused by an increased production of a platelet-reducing substance in the spleen. Splenectomy therefore should abolish this factor.

Troland and Lee<sup>30, 31</sup> in 1938 demonstrated that acetone extracts of three spleens of patients with Werlhof's disease decreased the platelet count sharply when injected into rabbits. They called the principle responsible for these changes "thrombocytopen." However, these workers were not able to recover thrombocytopen from normal organs (including the spleen), nor from spleens of patients with hemolytic anemia or Banti's syndrome.

Subsequently many investigators have studied this problem. Although some could confirm these findings,<sup>6, 13, 16, 17, 18, 22, 25</sup> others did not observe any platelet reduction<sup>11, 15, 19, 28</sup> or found inconsistent results.<sup>4, 7, 10, 12, 32, 35</sup> The variable responses were often considered to be caused by the mode of preparing the extracts. Most investigators used acetone, although Hobson and Witts<sup>10</sup> found a suspension of the tissue particles in Ringer solution more effective. Interpretation of the results was also difficult because of the spontaneous fluctuations of the platelet counts in the test animals. Most workers used rabbits, but rats<sup>4, 32, 35</sup> and dogs<sup>7</sup> also were employed.

Our approach to this problem was directed by the following considerations. Assuming that a thrombocytopen is manufactured by the spleen in thrombocytopenic purpura, it seems very likely that the liver—being the first organ to receive the blood coming from the spleen—might inactivate this principle. This assumption becomes even more probable when Moolten's hypothesis,<sup>13, 14</sup> that thrombocytopen may be a steroid, is taken into account. The effect of thrombocytopen may then depend not only on its concentration in the extract, but also

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on the modifying activity of the liver of the test animal. Consequently, a more reliable test for thrombocytopen may become available by injection of organ extracts into animals with liver cell damage.

These considerations seem to be borne out by our experimental studies. When organ homogenates were injected intraperitoneally into rats in which a high degree of liver cell damage was produced by carbon tetrachloride, a marked reduction of the platelet counts was consistently demonstrable. These results were not only obtained with preparations from normal and pathologic human spleens, but also with material from other normal animal organs. A thrombocytopen\* was found also in urine, from which it could be extracted with ether.

#### MATERIAL AND METHODS

All studies were performed on albino rats weighing between 150 and 300 grams. Male rats were used since males are more susceptible to intoxication with carbon tetrachloride than are females.<sup>8</sup> The animals were kept in separate metal cages and maintained on Purina dog chow and water. Each rat was used for one experiment only.

Blood was obtained by heart puncture, under ether anesthesia, with tuberculin syringes and gauge 23 needles, which had previously been rinsed with a 3.8 per cent sodium citrate solution. About 0.2 c.c. of blood was transferred into a small paraffin dish containing a few crystals of an ammonium and potassium oxalate mixture.<sup>9</sup> The blood was stirred gently with a paraffin stick to achieve equal distribution of the formed elements. Films were prepared from the last remaining drop of blood in the syringe.

In each instance the red and white cell counts were determined besides the enumeration of the platelets. For the latter, the method of Rees and Ecker<sup>10</sup> was used. The hemocytometers were kept for a period of twenty minutes in a wet chamber to assure proper settling of the thrombocytes without loss of fluid. All platelet counts were performed simultaneously by two workers and the results averaged.

Normal values established with these procedures are: R.B.C., 7.36 million  $\pm$  1.3; W.B.C., 16,300  $\pm$  6,000; platelets, 500,000 to 900,000 per cubic millimeter. Rarely an animal was encountered showing a platelet count below 500,000. Such rats were considered unsuitable for the experiments. Ether anesthesia did not influence the results.<sup>5</sup>

Evaluation of the platelet count must take into account that the number of thrombocytes in the same animal may vary greatly, the range lying between 500,000 and 900,000. The same figures represent the normal values found in the rat population as a whole. Normal animals used in this study never had a platelet level below half a million. Consequently a decrease was considered significant only when the thrombocytes were reduced below this value.

*Production of Liver Cell Damage.*—Carbon tetrachloride (0.5 c.c. per kilogram) was injected intraperitoneally on alternate days according to the method of Brauer and Root.<sup>11</sup> This procedure assures severe liver cell damage with only minor injury to the kidneys. Two injections were found to be sufficient to secure adequate responses to thrombocytopen. If desired, the liver cell damage may be maintained by repeated administrations of carbon tetrachloride. Blood films from such animals very frequently showed many target cells. Target cells are known to occur also in human parenchymatous liver disease.<sup>1</sup>

*Preparation of Organ Suspensions.*—After removal from the organism, the organs were immediately wrapped in a clean cloth and placed on dry ice in a deep freeze unit. For preparation of the saline suspension, the organs were defrosted, weighed, and cut into small pieces. These were mixed with double the volume of physiologic saline solution in a Waring Blender, gradually set to maximal speed for a period of ten minutes. The saline suspension

\*For the sake of convenience and because of usage in the literature the term "thrombocytopen" will be used throughout to denote a chemically unknown factor or factors which depress the platelet count in the test animals.

TABLE I. EFFECT OF DURACILIN AND OF CARBON TETRACHLORIDE ON THE PLATELET COUNT OF RATS

DRUG TESTED	NUMBER OF INJECTIONS	NUMBER OF RATS USED	AVERAGE COUNTS										AVERAGE % DECREASE OF PLATELETS	
			R.B.C. (MILLIONS)			W.B.C. (THOUSANDS)			PLATELETS (THOUSANDS)					
			BEFORE INJECT.	AFTER FIRST INJECT.	AFTER LAST INJECT.	BEFORE INJECT.	AFTER FIRST INJECT.	AFTER LAST INJECT.	BEFORE INJECT.	AFTER FIRST INJECT.	AFTER LAST INJECT.	BEFORE FIRST INJECT.	AFTER FIRST INJECT.	AFTER LAST INJECT.
Duracilin	1	4	7.4	7.1	-	13.2	11.8	-	655	776	-	None	-	
	2	4	7.4	7.1	6.9	20.2	16.5	16.1	668	792	830	None	None	
	(On consecutive days)													
	On alternate days:													
Carbon tetra-chloride	2	7	7.6	-	7.6	16.4	-	10.9	755	-	652	-	14	
	3	22	7.2	-	7.2	13.2	-	11.8	713	-	643	-	10	
	4	7	7.3	-	7.5	14.7	-	15.2	786	-	662	-	16	

was then filtered first through two layers and then through four folds of gauze. After this the suspension was put back into the freezing unit and defrosted completely for each particular use.

*Procedure for the Determination of the Effect of Organ Suspensions in the Rats With Liver Cell Damage.*—Liver cell damage was first produced by two applications of carbon tetrachloride. Two days following the last injection, the red cell, white cell, and platelet counts were determined. Then each animal was injected intraperitoneally with 10 c.c. of the organ suspension. Furthermore, carbon tetrachloride was given to maintain the liver injury. To prevent the development of any infection,<sup>27</sup> 0.1 c.c. of procaine penicillin (Duracillin, Lilly) was also injected subcutaneously. The next day the counts were repeated. If the effect was minimal, the animals were reinjected with the homogenate and also with Duracillin. Counts were determined again the following day.

For the evaluation of each suspension a group of at least four rats was used. Significant changes of the red and white cell counts were hardly ever noted. These findings preclude the interpretation that the observed reduction in the number of platelets was caused by hemodilution. Occasionally the organ suspension was quite toxic and the animals died within a few hours after the injections.

The platelet counts of each group of rats before and after the injections were averaged and the decrease was expressed in per cent of the average platelet count. By this procedure the individual variation in the reaction of the animals toward the active principle contained in the material were taken into account.

## RESULTS

1. *Effect of Penicillin and of Carbon Tetrachloride on the Platelet Count.*—Procaine penicillin (Duracillin, 0.1 c.c.), which was used prophylactically throughout the experiments, does not produce thrombocytopenia. (Table I.) The apparent increase of the platelet level lies within the range of daily fluctuations.

Carbon tetrachloride was tested in thirty-six animals. The slight decline in the average platelet count (Table I) is insignificant since no animal showed a decrease below the range of normal, even if four injections of the liver poison were given. Both drugs did not influence the red and white cell counts significantly.

2. *Comparison of the Effect of Injected Organ Suspensions on the Platelet Counts of Normal Rats and Rats With Liver Cell Damage.*—Seven organ suspensions were studied. Ten cubic centimeters of the homogenized organs were injected in each instance. Table II summarizes the values obtained. Each value represents the average platelet count of at least four animals.

The organ suspensions, when injected into normal rats, left the platelet level practically unchanged with the exception of the material prepared from normal dog kidneys. In this experiment (Experiment 7) the first injection brought the thrombocyte count definitely out of the normal range and a second injection produced a further considerable decrease.

In the rats with liver cell damage, the first injection consistently lowered the platelet counts significantly. Following a second injection the decrease was still more pronounced, with the exception of Experiment 4 in which the platelet level returned to normal. This was the only observation of this kind encountered in this study. Ordinarily a second injection of potent material, when given to rats with liver cell damage, either maintains or even further decreases the thrombocytes in the circulation.

TABLE II. COMPARISON OF THE EFFECT OF ORGAN HOMOGENATES ON THE PLATELET COUNT OF NORMAL RATS AND OF RATS WITH LIVER CELL DAMAGE\*

EX- PERI- MENT	ORGAN	LIVER CELL DAMAGED RATS					NORMAL RATS				
		AVERAGE PLATELET COUNT (THOUSANDS)			AVERAGE % DECREASE OF PLATELETS		AVERAGE PLATELET COUNT (THOUSANDS)			AVERAGE % DECREASE OF PLATELETS	
		BE- FORE IN- JECT.	AFTER FIRST INJECT.	AFTER SECOND INJECT.	AFTER FIRST INJECT.	AFTER SECOND INJECT.	BE- FORE IN- JECT.	AFTER FIRST INJECT.	AFTER SECOND INJECT.	AFTER FIRST INJECT.	AFTER SECOND INJECT.
1	Human spleen (Idiopathic thrombocytopenic purpura)	660	376	—	43	—	749	689	—	8	—
2	Human spleen (Hodgkin's dis- ease with symp- tomatic thrombo- cytopenic pur- pura)	670	247	—	63	—	590	622	666	None	None
3	Human spleen (Lymphosarcoma with symptoma- tic thrombo- cytopenic pur- pura)	630	370	295	41	53	759	674	790	11	None
4	Human spleen (Splenic vein thrombosis)	612	341	527	44	14	714	650	610	9	15
5	Human spleen (Paroxysmal nocturnal hemo- globinuria)	600	236	—	61	—	718	—	728	—	None
6	Dog spleen (Normal)	615	354	247	42	60	614	676	620	None	None
7	Dog kidney (Normal)	599	212	—	65	—	704	476	186	32	74

\*Each value represents the average of at least four animals.

The findings seem to indicate that liver cell damage permits a principle in the organ suspensions to exert its effect on the platelet level. Apparently the normal liver renders this thrombocytopenic agent ineffective. Contrary to our expectations, however, the suspensions prepared from the spleens of patients with normal platelet counts (Experiments 4 and 5) seemed to be as potent as those obtained from the spleens of patients with severe thrombocytopenic purpura (Experiments 1, 2, and 3). Furthermore, thrombocytopen was equally demonstrable in the normal dog spleen and was apparently present in the highest concentration in normal dog kidneys, since the latter material produced thrombocytopenia even in normal rats.

At this point in our investigation it was decided to use only rats with liver cell damage as test animals in further experiments. It also was felt that to secure the evidence so far obtained, more organ suspensions of various types should be tested for the presence of the thrombocytopenic principle.

3. *Effect of Injected Homogenized Normal and Pathologic Spleens on the Platelet Count of Rats With Liver Cell Damage.*—Table III summarizes the results.

TABLE III. EFFECT OF HOMOGENIZED NORMAL AND PATHOLOGIC HUMAN SPLEENS ON THE PLATELET COUNT OF RATS WITH LIVER CELL DAMAGE\*

EXPERIMENT	DIAGNOSIS	AVERAGE COUNTS						AVERAGE % DECREASE OF PLATELETS			
		R.B.C. (MILLIONS)		W.B.C. (THOUSANDS)		PLATELETS (THOUSANDS)		BEFORE FIRST INJECT.		AFTER SECOND INJECT.	
		BEFORE INJECT.	AFTER FIRST INJECT.	BEFORE INJECT.	AFTER FIRST INJECT.	BEFORE INJECT.	AFTER FIRST INJECT.	BEFORE INJECT.	AFTER FIRST INJECT.	BEFORE INJECT.	AFTER FIRST INJECT.
1	Traumatic rupture (normal spleen)	7.0	7.3	-	-	18.1	18.6	582	283	-	51
2	Hyperinsulinism (normal spleen)	7.0	7.4	-	-	19.2	18.2	554	197	-	64
3	Idiopathic thrombocytopenic purpura	6.9	5.7	-	-	11.9	14.9	600	376	-	43
4	Idiopathic thrombocytopenic purpura	7.4	7.2	7.3	16.1	18.0	19.6	546	350	207	36
5	Hodgkin's disease (symptomatic thrombocytopenic purpura)	7.1	6.9	-	-	12.1	10.7	670	247	-	63
6	Lymphosarcoma (symptomatic thrombocytopenic purpura)	7.8	8.2	7.4	17.4	15.9	14.1	630	370	205	41
7	Banti's syndrome (symptomatic thrombocytopenic purpura)	7.8	7.5	-	-	20.7	19.1	697	344	-	51
8	Liver cirrhosis (symptomatic thrombocytopenic purpura)	7.1	7.6	-	-	17.6	18.0	733	468	381	36
9	Thrombotic thrombocytopenic purpura	7.4	6.3	-	-	21.3	12.7	635	351	-	45
10	Splenic vein thrombosis	7.7	7.7	7.5	22.6	17.5	19.7	612	341	527	44
11	Hodgkin's disease	7.4	7.3	-	-	16.6	16.9	655	366	-	14
12	Gaucher's disease	7.5	7.4	-	-	17.8	17.6	657	314	-	52
13	Acquired spherocytic hemolytic anemia	7.8	7.5	7.4	17.2	14.9	16.1	618	522	389	20
14	Acquired spherocytic hemolytic anemia	7.3	7.2	7.3	18.5	17.8	17.3	616	414	334	33
15	Congenital spherocytic hemolytic anemia	7.5	7.4	7.4	17.2	15.9	15.2	617	382	380	38
16	Paroxysmal nocturnal hemoglobinuria	7.4	7.3	-	-	17.1	17.4	600	236	-	61

\*Each value represents the average of at least four animals.



All spleens were obtained during life with the exception of the case of thrombotic thrombocytopenic purpura.<sup>26</sup> In this instance the organ was received one hour after the death of the patient. The few values previously mentioned in Table II, are included in this summary for a more convenient survey of the whole material studied. Regardless of the weight of the spleen and the presence of pathologic tissues (Hodgkin's granuloma, lymphosarcoma, Gaucher's disease, etc.), 10 c.c. of the homogenized suspension were always injected. As can be seen from Table III, all suspensions lowered the platelet level considerably. Usually a single injection produced a reduction of more than 40 per cent. If the decrease was less than 40 per cent of the preinjection level, a second injection was given, which in most instances caused a further decline of the thrombocyte count. The spleens of the patients with spherocytic hemolytic anemias seemed to contain comparatively less of the thrombocytopenic agent, although it still was readily demonstrable. Suspensions from the normal spleens caused approximately the same reduction of the level of thrombocytes as could be achieved with some suspensions prepared from pathologic spleens. No correlations were demonstrable between platelet reduction in the patient whose spleen was used for the injection and the degree of platelet decline in the injected animals. Although various pathologic spleens were used in this series of experiments, none of the homogenates produced any significant changes in the red or white cell counts.

4. *Effect of Normal Organ Homogenates on the Platelet Count of Rats With Liver Cell Damage.*—The results are compiled in Table IV. The values show convincingly that the thrombocytopenic agent is not only present in dog and beef spleen, but may also be found in homogenates prepared from lungs, heart, and kidneys of a healthy dog. The suspension of lung tissue caused the severest depression of the platelet level, namely a reduction of 75 per cent of the original count.

TABLE IV. EFFECT OF SUSPENSIONS OF NORMAL ANIMAL ORGANS ON THE PLATELET COUNT OF RATS WITH LIVER CELL DAMAGE\*

ORGAN SUSPENSION PREPARED FROM	AVERAGE PLATELET COUNT (THOUSANDS)			AVERAGE % DECREASE OF PLATELETS	
	BEFORE INJECT.	AFTER FIRST INJECT.	AFTER SECOND INJECT.	AFTER FIRST INJECT.	AFTER SECOND INJECT.
Dog spleen 1	615	354	247	42	60
2	616	247	198	60	68
3	593	377	286	36	52
Dog kidney 1	599	212	—	65	—
2	595	231	210	61	64
Dog heart	660	286	—	57	—
Dog lung	613	156	—	75	—
Beef spleen	623	411	338	34	46

\*Each value represents the average of at least four animals.

5. *The Dose-Response Relationship Between Injected Organ Suspensions and Reduction of the Platelet Count.*—For the evaluation of our findings the problem is of obvious importance whether some correlation exists between the amount of homogenate injected and the demonstrable fall of the platelet level.

Doses of 10, 8, 6, 4, 2, and 1 c.c. of the same saline suspension prepared from a normal human spleen were injected into groups of at least four rats with liver cell damage. The results are compiled in Table V.

TABLE V. DOSE-RESPONSE RELATIONSHIP BETWEEN AMOUNT OF HOMOGENATE INJECTED AND DECLINE OF PLATELET COUNT

AMOUNT OF SUSPENSION (NORMAL HUMAN SPLEEN) INJECTED (C.C.)	RATS USED	AVERAGE % DECREASE OF PLATELETS
10	4	64 $\pm$ 12
8	4	61 $\pm$ 22
6	5	41 $\pm$ 13
4	4	35 $\pm$ 4
2	4	43 $\pm$ 11
1	4	24 $\pm$ 24

As can be seen from the table, the two largest doses (10 and 8 c.c.) produced the greatest decline of the platelet count, 64 and 61 per cent respectively. However, the response to 2, 4, and 6 c.c. of the suspension was approximately the same (35 to 43 per cent). The smallest dose of 1 c.c. elicited also the slightest effect, i.e. 24 per cent.

These results show that the dose-response relationship is not a linear one, but that, within certain ranges, larger amounts of the suspension produce greater responses. Since these studies were performed with crude homogenates, it was not considered advisable to pursue the problem further. With the isolation and purification of thromboeytopen, the rat with liver cell damage promises to become a reliable test animal for the quantitative bio-assay of the platelet-reducing agent.

6. *Effect of Hemoglobin Solutions and of Thromboplastin on the Platelet Count of Rats With Liver Cell Damage.*—It was noticed that many animals developed hemoglobinuria following the injection of the organ homogenates. In the course of the freezing and defrosting processes employed in the preparation of the suspensions, the blood contained in the organs becomes hemolyzed. Thus a relatively large quantity of free hemoglobin and erythrocyte stroma was injected into the rats with liver cell damage together with the homogenized tissues. The question arose whether the thromboeytopenia in the animals might not be caused by this hemoglobinemia. In cases of severe hemoglobinemia in human beings, regardless of the etiology, thromboeytopenia is frequently observed.<sup>24</sup>

Therefore, 10 c.c. of a 3 per cent suspension of washed dog erythrocytes hemolyzed by addition of distilled water, were injected into the animals. As can be seen from Table VI (Experiments 1 and 2), this procedure was followed by an insignificant drop of the thromboeyte level.

The very conspicuous reduction of the platelet level with the lung suspension (Table IV) led us to consider the possibility that thromboplastin may be identical with the thromboeytopenic agent. Thromboplastin is another ubiquitous tissue factor, known to be present in particularly high concentration in the lungs. Consequently Thromboplastin Maltine, a rabbit lung preparation, as well as rabbit brain thromboplastin, made according to Quick's method,<sup>25</sup>

TABLE VI. EFFECT OF HEMOGLOBIN SOLUTIONS AND OF THROMBOPLASTIN ON THE PLATELET COUNT OF RATS WITH LIVER CELL DAMAGE\*

EXPERIMENT	PREPARATION INJECTED	AVERAGE PLATELET COUNT (THOUSANDS)			AVERAGE % DECREASE OF PLATELETS	
		BEFORE INJECT.	AFTER FIRST INJECT.	AFTER SECOND INJECT.	AFTER FIRST INJECT.	AFTER SECOND INJECT.
1	Laked dog blood	636	550	-	13	-
2	Laked dog blood	630	-	545	-	13
3	(a) Suspension of dried rabbit lung	583	178	-	69	-
	(b) Milky supernatant fluid (rich in thromboplastin)	604	468	-	22	-
	(c) Granular residue (poor in thromboplastin)	517	161	-	69	-
4	(a) Suspension of dried rabbit brain	545	323	-	40	-
	(b) Milky supernatant fluid (rich in thromboplastin)	576	409	-	29	-
	(c) Granular residue (poor in thromboplastin)	539	256	-	53	-

\*Each value represents the average of at least four animals.

was investigated. For each single rat experiment, 200 mg. of the lung or brain powder were first suspended in saline and then injected as a whole. In another experiment the same type of organ emulsion was incubated at 56° C. for fifteen minutes and then centrifuged at low speed for five minutes. The supernatant milky fluid was injected separately, as was a suspension of the granular material found at the bottom of the centrifuge tube. Determination of the prothrombin times revealed that the former contained much more thromboplastin than the latter (13 and 30 seconds respectively).

The results of these experiments may be seen in Table VI (Experiments 3 and 4). Since the supernatant fluid, richer in thromboplastin, is evidently poorer in thrombocytopen, the conclusion seems justified that these two factors are not identical.

These experiments furthermore show that thrombocytopen cannot be extracted satisfactorily from organs by means of acetone since the thromboplastin preparations were made by macerating the organs under acetone and discarding the acetone extract. However, the remaining dry powder still contained a very potent thrombocytopenic agent.

*7. Effect of Normal Urine and the Urine of Patients With Thrombocytopenic Purpura on the Platelet Count of Rats With Liver Cell Damage.*—Our experiments have demonstrated that thrombocytopen is inactivated by the liver. Inactivation of metabolites often occurs by conjugation with acids in the liver and subsequent excretion of the substances in the urine. When 10 c.c. of the concentrated morning urine of two healthy male persons were injected into rats with liver cell damage, definite reductions of the platelet counts were noticeable. The same results were obtained with urine specimens from two female patients with severe thrombocytopenic purpura.

The thrombocytopenic agent could be extracted from the urine specimens with ether. Extraction was performed by adding 3 parts of ether to 1 part of urine in a separatory funnel and shaking for ten minutes. This procedure then was repeated twice. From the combined extracts the solvent was evaporated at room temperature and the dark and sticky residue was dissolved in the appropriate amounts of carbon tetrachloride needed for maintaining the liver cell damage in the rats. By addition of saline, a good emulsion could be obtained with shaking, which permitted the application of the correct doses of the various substances. It should be emphasized that no change of the platelet count was seen when carbon tetrachloride and the thrombocytopen-free urine residue were injected (Table VII).

The demonstration that the platelet reducing agent can be completely recovered from the urine with ether will probably permit quantitative estimations of the urinary output of this factor in normal and various pathologic conditions. Such studies will be reported later. So far the experiments summarized in Table VII merely show that thrombocytopen is excreted in the urine.

When the ether extract of larger amounts of urine was injected into *normal* rats (extract residue of 175 c.c. of pooled urine, suspended in saline, injected per rat), no significant reduction of the platelet level was observed. These results are in agreement with the findings of Tocantins,<sup>28</sup> who could not decrease the thrombocytes of normal rabbits with urinary extracts. The detoxifying action of the normal liver apparently is very efficient. The amount of thrombocytopen able to reduce the platelet count in normal animals remains to be determined. Such studies may better be performed when sufficient quantities of the purified material are available.

8. *Mode of Action of Thrombocytopen*.—Although the reduction of thrombocytes following the injection of organ homogenates is sometimes a considerable one, we have never noticed the development of true purpuric lesions in our experimental animals. This finding is not an unexpected one. Roskam<sup>23</sup> as well as Bedson<sup>2</sup> showed that by intravenous injection of gelatin or agar extreme thrombocytopenia may be produced without any increased bleeding tendency. However, if the capillaries were damaged simultaneously (e.g. by anti-red cell serum), purpura resulted.

From the experimental observations of Torrioli and Puddu,<sup>29</sup> it is likely that thrombocytopen acts directly on the megakaryocytes by inhibiting platelet production. This view is also emphasized by Dameshek and Estren.<sup>7</sup> We have postponed a systematic study of the marrow until a purified thrombocytopen is at hand. In the few instances in which the marrow was examined after injection of organ homogenates, a slight increase of the more immature basophilic megakaryocytes was noted occasionally.

In a few animals the recovery phase following a single injection of an organ homogenate was studied. If after the production of thrombocytopenia all applications were stopped, the platelet count returned to normal within twenty-four to seventy-two hours.

TABLE VII. EFFECT OF URINE AND OF URINE EXTRACTS ON THE PLATELET COUNT OF RATS WITH LIVER CELL DAMAGE\*

TYPE OF URINE	FRESH URINE						URINE AFTER REMOVAL OF THROMBOCYTOGEN WITH ETHER						ETHER EXTRACT OF URINE					
	PLATELET COUNT (THOUSANDS)			AVERAGE % DECREASE OF PLATELETS			PLATELET COUNT (THOUSANDS)			AVERAGE % DECREASE OF PLATELETS			PLATELET COUNT (THOUSANDS)			AVERAGE % DECREASE OF PLATELETS		
	BEFORE INJECT.	AFTER FIRST INJECT.	AFTER SECOND INJECT.	BEFORE INJECT.	AFTER FIRST INJECT.	AFTER SECOND INJECT.	BEFORE INJECT.	AFTER FIRST INJECT.	AFTER SECOND INJECT.	BEFORE INJECT.	AFTER FIRST INJECT.	AFTER SECOND INJECT.	BEFORE INJECT.	AFTER FIRST INJECT.	AFTER SECOND INJECT.	BEFORE INJECT.	AFTER FIRST INJECT.	AFTER SECOND INJECT.
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Normal	579	406	-	30	26	58	543	536	547	1	0	0	544	372	252	31	54	
	585	432	244	26	26	58	523	529	460	0	11.	11.	545	370	240	32	56	
Thrombocytopenic purpura	593	387	272	35	35	54	554	550	577	0	0	0	553	285	331	48	40	
	565	363	-	36	36	-	583	548	562	6	4	4	566	359	372	37	34	

\*Each value represents the average of at least four animals.

## DISCUSSION

The results of our experiments seem to indicate that a thrombocytopenic agent can be obtained from many different normal organs (spleen, lungs, heart, kidneys, and brain). Since consistent lowering of the platelet count is demonstrable only in animals with liver cell damage and since it also can be produced by the injection of urine, one may speculate that physiologically thrombocytopen is rendered innocuous in the liver and then is excreted in the urine. Since it can be recovered from the ether extract of the urine, this factor is probably lipid in nature. Thus the metabolic behavior of thrombocytopen resembles closely the well-known pattern found with many steroid compounds.

The role which the liver plays in the inactivation of thrombocytopen may also partly explain the inconsistent or negative results of many investigators (ten out of nineteen) who worked with organ or urine extracts and injected them into normal animals. Our results permit the statement that the rat with liver cell damage is well suited for the demonstration of the thrombocytopenic agent.

Thrombocytopen has been of interest to most investigators on account of its hypothetical role in the pathogenesis of thrombocytopenic purpura. Torrioli and Puddu<sup>29</sup> have emphasized that the principle which injured the megakaryocytes in bone marrow cultures is also present in many normal organs. If thrombocytopen is identical with this factor of the Italian authors, who used a "protein-free aqueous extract," our findings confirm their observations. We have, however, not been able to demonstrate any significant differences in the degree of thrombocytopenia produced in the animals by the homogenates prepared from the various types of pathologic spleens. The results obtained with the sixteen human spleens used in this study (Table III) may be grouped in relation to the hematologic status of the splenectomized patients. With normal spleens the average platelet reduction in the animals following a single injection was 57.5 per cent; with spleens from patients with severe idiopathic or symptomatic thrombocytopenic purpura (Table III, Experiments 3 to 9) the decrease was 45 per cent; and with spleens of patients without thrombocytopenia but with other hematologic disorders (Table III, Experiments 10 to 16) the lowering of the platelets amounted to 41.7 per cent.

Since the suspensions always were prepared in the same manner and the injected dose was kept constant, these results indicate that the concentration of the platelet-reducing principle per gram of tissue apparently was not increased in the spleens from patients with thrombocytopenic purpura. Furthermore, there was no splenomegaly in the patients with Werlhof's disease, which fact rules out the possibility that the absolute amount of thrombocytopen within these extirpated organs may have been abnormally large.

On the other hand it must be realized that crude homogenates are not too well suited for exact quantitative evaluation, as may be seen from our study of the dose-response relationship (Table V) in which a saline suspension prepared from a normal spleen was used. Furthermore, the quantity of a biologic agent present in an organ at any given moment does not directly reflect on its

production within this organ, or on its release into the circulation. Since our demonstration of the thrombocytopenic principle in the urine did not include any quantitative estimation of the output per day, this problem requires further investigation. Nevertheless, if one takes into consideration that so many normal organs contain at least a similar concentration of thrombocytopen as can be found in normal as well as in pathologic spleens, there is no evidence available from our data to support the hypothesis that this agent may definitely be involved in the pathogenesis of thrombocytopenic purpura. However, we are not yet able to explain why some investigators, although using normal animals for testing, could demonstrate the principle more readily in pathologic spleens than in normal organs.

In this respect the work of Moolten<sup>13, 11</sup> is of particular interest. This author reported that normal and also certain pathologic spleens (e.g. in Hodgkin's disease) contain not only thrombocytopen but also another lipoid factor which has the opposite effect on the platelet level. He called this antagonist of thrombocytopen, capable of increasing the thrombocyte count, "thrombocytosin." Both thrombocytopen and thrombocytosin could be obtained as relatively purified substances and formed ether-insoluble digitonides in the manner of steroids. Thrombocytosin also was found in high concentrations in fatty tissues and in egg yolk and was effective when given orally. Normal urine and particularly the urine of a splenectomized patient sometimes contained large amounts of the platelet-increasing agent. Moolten speculates that thrombocytopen may be concerned in balancing the thrombocytosis produced by the dietary factor thrombocytosin. Under the conditions of our experiments with rats with liver cell damage we have not yet encountered any results suggestive of an effect of thrombocytosin.

Ungar<sup>33, 34</sup> has isolated two substances from the spleen capable of influencing the bleeding time of guinea pigs. "Splenin A" decreases, whereas "Splenin B" definitely increases, the bleeding time. Splenin B is considered to be possibly identical with the thrombocytopen of Troland and Lee.<sup>31</sup> However, no experimental evidence is yet available demonstrating the ability of Splenin B to reduce the platelet level. It should be of interest to test these substances in rats with liver cell damage.

So far we have assumed that the thrombocytopenic agents found in the various organ homogenates as well as in the urine are one and the same principle. There is actually no proof for such an assumption, and this approach should be considered primarily a working hypothesis. We are, however, convinced that the thrombocytopenia in the rats with liver cell damage is a specific response to a single factor or to several factors. Such an interpretation is supported by the finding that the reduction of the platelets was not accompanied by any change in red and white cell counts.

The physiologic significance of thrombocytopen is at present unknown. It should be kept in mind that the demonstration of a powerful biologic agent obtained from animal tissues does not necessarily indicate any "physiologic activity." Heparin, for instance, is certainly a very potent anticoagulant, but its role in the normal coagulation process is still debatable.

The use of the rat with liver cell damage should provide a reliable tool for the study of many problems related to "thrombocytopen." Whatever the outcome may be, the doubts concerning the reality of such a factor or factors may now be abandoned. Their existence at least seems to be assured.

#### SUMMARY

1. Thrombocytopen is the name which has been given to a principle, present in the spleen which, when injected into laboratory animals, is said to decrease their platelet count. This principle is believed to play an important role in the pathogenesis of thrombocytopenic purpura. So far nine out of nineteen investigators have confirmed the existence of this thrombocytopenic agent, whereas the others obtained either inconsistent or negative results.

2. Our approach to this problem was directed by the following considerations. Assuming that thrombocytopen is manufactured predominantly by the spleen, it seems very likely that the liver—being the first organ to receive blood coming from the spleen—may be involved in the inactivation of this principle. The effect of thrombocytopen may then depend not only on its concentration in the organ extracts but also on the modifying activity of the liver in the test animals. Consequently, a more reliable test for thrombocytopen may become available by injection of organ suspensions into animals with liver cell damage.

3. When organ homogenates were injected into rats with a high degree of liver cell damage produced by carbon tetrachloride, a considerable drop of the platelet count was consistently produced. The same organ suspensions did not reduce the platelet count of normal animals. Neither penicillin (used for prevention of infection) nor carbon tetrachloride by themselves influenced the platelet levels. No changes in the red or the white cell counts were observed.

4. Two normal human spleens, seven spleens from patients with thrombocytopenic purpura (idiopathic and symptomatic), and seven spleens from patients without any thrombocytopenia, but with other hematologic disorders, were tested. All these organs contained thrombocytopen in approximately similar concentrations. The platelet-reducing agent also was found in at least the same amount in normal organs (lung, heart, kidney, brain) obtained from dogs, cattle, or rabbits.

5. When urine specimens of normal individuals or from patients with thrombocytopenic purpura were injected into rats with liver cell damage, a significant reduction of the platelet level also occurred. The platelet-reducing agent could be extracted with ether.

6. These results may indicate that physiologically thrombocytopen is rendered innocuous in the liver and then excreted in the urine. Since it can be recovered from the ether extract, this factor is probably lipid in nature. Thus the metabolic behavior of the thrombocytopenic agent resembles the well-known pattern of steroid compounds.

7. There is at present no evidence available from our data which would support the hypothesis that thrombocytopen is definitely involved in the pathogenesis of thrombocytopenic purpura. However, further studies are necessary.



production within this organ, or on its release into the circulation. Since our demonstration of the thrombocytopenic principle in the urine did not include any quantitative estimation of the output per day, this problem requires further investigation. Nevertheless, if one takes into consideration that so many normal organs contain at least a similar concentration of thrombocytopen as can be found in normal as well as in pathologic spleens, there is no evidence available from our data to support the hypothesis that this agent may definitely be involved in the pathogenesis of thrombocytopenic purpura. However, we are not yet able to explain why some investigators, although using normal animals for testing, could demonstrate the principle more readily in pathologic spleens than in normal organs.

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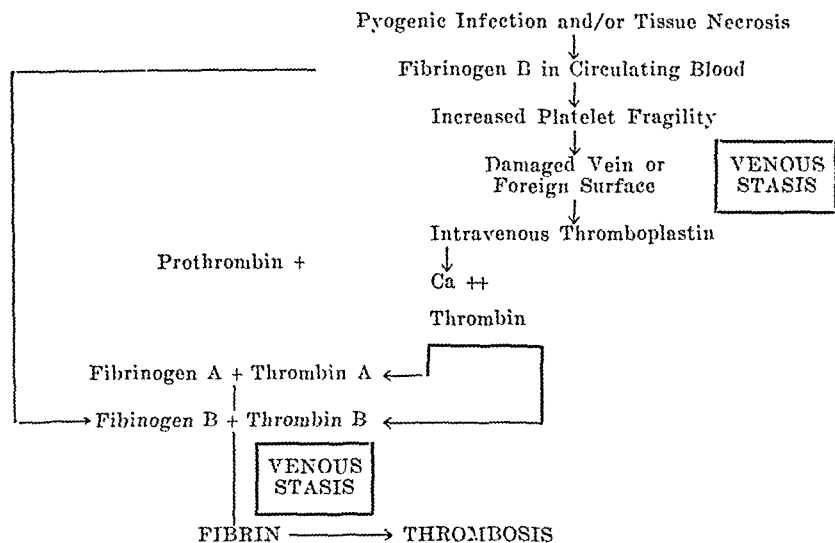
# THE FIBRINOGEN B TEST AND INTRAVASCULAR THROMBOSIS

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IN RECENT years intravascular thrombosis has assumed a prominent position on the list of postoperative complications as other more common complications have been brought under control. A laboratory method for determining the presymptomatic thrombosis would be of great value in reducing the mortality rate and serious sequelae of intravascular thrombosis. Our interest in the fibrinogen B reaction was stimulated by the possible relationship it might hold to the diagnosis of intravascular thrombosis. The fibrinogen B reaction described by Cummine and Lyons<sup>1</sup> was studied for a six-month period on the Surgical Service of Brooke General Hospital. The data set down in the report were derived from patients who had been hospitalized during this period of study.

Cummine and Lyons accepted the earlier hypothesis of Berquist,<sup>1</sup> who stated that just prior to the formation of an intravascular thrombus a "pre-thrombotic state" exists during which there is a significant reduction in venous clotting time. Although these investigators employed slightly different methods of clotting time determination, the critical zone was considered to be 3 to 4 minutes, or less.

Cummine and Lyons introduce the concept of an intermediary product in the fibrinogen-to-fibrin reaction, to which they give the name fibrinogen B. The presence of fibrinogen B in the circulating plasma is considered abnormal. They correlate its presence in the plasma with the production of intravascular thrombosis. They note that fibrinogen B appears in the plasma whenever tissue necrosis is present, especially in the case of pyogenic infection. Lyons has demonstrated in vitro that platelet fragility increases in the presence of fibrinogen B and has postulated the following possible mechanism<sup>1</sup>:



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Cummine and Lyons studied 580 patients and catalogued the results of their investigation as follows<sup>1</sup>:

Group 1.—In these patients free fibrinogen B does not occur in the plasma and the coagulation graph is of normal type. Intravascular thrombosis does not develop in these cases.

Group 2.—Free fibrinogen B may be found in the plasma, either persistently or intermittently, but the coagulation graph is of normal type. Providing that no previous thrombosis has occurred and is remaining as an intravascular foreign body, and that venous stasis is prevented, thrombosis does not develop in these cases.

Group 3.—Free fibrinogen B occurs in the plasma and the coagulation times remain persistently low in the order of 3 to 4 minutes. Intravascular thrombosis appears to be inevitable in these cases, either in the legs or at the lung bases.

Group 4.—No free fibrinogen B is found in the plasma but the coagulation graph is consistently low. This combination of the two factors has not occurred in any of the patients studied as a primary phenomenon, but has been seen on several occasions after an intravascular thrombosis has occurred. In each instance it is significant that the thrombosis recurred at the initial site and can be interpreted as a local exacerbation due to the presence of an intravascular foreign body in association with venous stasis.

We have undertaken a study for the purpose of estimating the practical value of the fibrinogen B test in a military medical establishment.

#### METHODS AND MATERIALS

*Clotting time determinations* were performed after the method outlined by Cummine and Lyons with only slight modification, i.e., the capillary tube filled with fingertip blood was broken every thirty seconds rather than every sixty seconds during the first four minutes.

*Fibrinogen B determinations* were performed as follows. The reagent was prepared by dissolving 2 Gm.  $\beta$ -naphthol in 100 ml. of 50 per cent alcohol. If the solution turned brown, it was exposed to oxygen in order to return it to the colorless state. Blood was withdrawn from the antecubital vein in a dry sterile syringe and 4.5 ml. were mixed immediately with 0.5 ml. of 1.1 per cent solution sodium oxalate. The blood sample was taken usually midway between breakfast and lunch; however, we found no evidence to suggest that the results were influenced by food intake. The specimen was centrifuged and 1 ml. plasma was placed in a 13 by 100 mm. tube. Five drops of the reagent were added to the plasma and the mixture was allowed to stand at room temperature for ten minutes. If a gel formed, a value of from 1 to 4+ was assigned, depending on the quantity of gel present. If no gel formed during that period, the test was recorded as negative for fibrinogen B.

#### RESULTS

A total of 553 fibrinogen B determinations and 337 capillary clotting time determinations were made on forty-eight hospital patients and four normal subjects. The maximum length of time an individual case was followed was fifty days, and the minimum, one day.

The cases chosen were selected deliberately for anticipated demonstration of a wide latitude of response to the test. Table I lists, by diagnosis, the types of cases studied and the results of the survey in terms of "positive," "doubtful," and "negative." The extent or severity of the pathologic changes is not correlated, nor is any quantitative estimate of the amount or duration of fibrinogen B present stated. In general, however, the quantity of fibrinogen B present was in direct proportion to the severity of the disease.

Columns three and four show the results obtained at room temperature and at 6° C. The description of the test set down by Lyons states that room temperature is to be used. We noted that certain tests negative at room temperature would become positive if repeated at 6° C. On further investigation, it was demonstrated that as the quantity of fibrinogen B rose it could be detected earliest at 6° C. and then later at room temperature. Conversely, as the plasma content of fibrinogen B fell, the test first became negative at room temperature and later at 6° C. Correctly or not, we assumed that the gel formations at room temperature and at 6° C. represented the same substance, and we used this finding as a rough quantitative index.

TABLE I

DIAGNOSIS	NUMBER OF CASES	RESULTS					
		ROOM TEMPERATURE			6° c.		
		P	D	N	P	D	N
Normal subjects	4	0	0	4	0	1	3
Thrombophlebitis, deep	12	3	4	5	11	0	1
Thrombophlebitis, superficial	1	0	0	1	1	0	0
Soft tissue trauma	2	1	0	1	2	0	0
Acute infections	6	0	1	5	5	0	1
Acute thermal and chemical burns	3	1	0	2	2	0	1
Chronic infection	9	0	1	8	6	3	0
Carcinoma (brain, stomach, cervix)	3	0	0	3	3	0	0
Abdominal stab wounds, contaminated	2	1	0	1	2	0	0
Uncomplicated postoperatives	5	1	0	4	2	3	0
Cirrhosis, periportal	1	0	0	1	1	0	0
Common duct obstruction	1	1	0	0	1	0	0
Myocardial infarction	1	0	0	1	1	0	0
Congestive failure	1	0	0	1	1	0	0
Late pregnancy with pyrexia (unknown origin)	1	0	0	1	1	0	0
Prolonged bed rest with hip fracture	1	0	0	1	0	1	0

P, positive; D, doubtful; N, negative.

Examination of the results in Table I indicates that there is no specificity of response peculiar to cases of intravascular thrombosis. Suggestive trends are poorly defined. One finding is outstanding, namely, that whenever tissue necrosis is present, the test is usually positive.

In the twelve cases of deep thrombophlebitis, only one patient demonstrated a positive test at room temperature for fibrinogen B on the day of clinical diagnosis. Subsequent positive results at room temperature were inconstant and afforded little recognized clinical value. In this series three of the twelve patients gave a clotting time of 4 minutes or less on the day of clinical diagnosis.

In the entire series of fifty-two cases, only two patients fulfilled one of the criteria of Cummine and Lyons, i.e., lowering of clotting time and the appearance of fibrinogen B in the plasma as indicative of inevitable thrombosis. Neither patient developed clinical symptoms of intravascular thrombosis.

#### DISCUSSION AND SUMMARY

Over a six-month period at Brooke General Hospital, the incidence of deep thrombophlebitis on the Surgical Service was less than 1 per cent. To conduct a survey for the purpose of extensively confirming or denying the hypothesis of

Cummine and Lyons, it would be necessary to follow daily every individual admitted to the Surgical Service, that is, to make in the neighborhood of 20,000 to 25,000 tests. In the experience reported herein, 553 tests were made. In only two instances the criteria of Cummine and Lyons for inevitable thrombosis were fulfilled, but neither patient developed clinical evidence for intravascular thrombosis. On the basis of experience to date, the test would appear to be of no particular value in the confirmation of a diagnosis of thrombophlebitis suspected clinically. While the present small series neither confirms nor denies the concepts expressed by Cummine and Lyons, it places some doubt on the precision of interpretation of low clotting times.

In light of the present good results of anticoagulant therapy in intravascular thrombosis initiated on clinical evidence alone, the practicality of this test is open to question as a routine examination on all surgical patients.

The fact that the test is more sensitive at 6° C. is not explained. The results are interesting but are difficult to assess.

We are in agreement with Cummine and Lyons in noting an apparent relation between the presence of tissue necrosis and the appearance of fibrinogen B in the circulating plasma. In fact, this relationship is the only constant finding. We have noted also, as have Cummine and Lyons, that there is a persistently negative test for fibrinogen B in thrombophlebitis after it is initially positive.

#### ADDENDUM

Dunn, Jackson, and Lyons<sup>2</sup> report a high percentage of positive fibrinogen B tests in congestive cardiac failure (97 per cent), recent coronary occlusion (100 per cent), thrombotic states (100 per cent), acute sepsis (100 per cent), and chronic sepsis (90 per cent).

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*Lyophilized Prothrombin-Free Plasmas.*—Using the method of Flosdorf and Mudd<sup>5</sup> a large quantity of prothrombin-free normal human pooled plasma was divided into 1.0 ml. aliquots, quick frozen at  $-70^{\circ}\text{C}$ . in a bath of dry ice and absolute alcohol, and dried by the lyophile process. The ampules were then sealed with an oxygen- $\text{CO}_2$  torch and stored in a deep freeze at  $-20^{\circ}\text{C}$ . until ready for use. This method of lyophilization, when properly performed, removes over 99 per cent of the water.<sup>5</sup> Reconstitution was effected by adding to the dried material 1.0 ml. of distilled water containing 10 per cent by volume of imidazole buffer solution (see below).

Prothrombin-free plasma (bovine) was lyophilized, stored, and reconstituted in a similar manner.

*Determination of the Stability of Stored Prothrombin-Free Plasmas.*—Determination of prothrombin concentration by the dilution method of Rosenfield and Tuft<sup>14</sup> was used as a measure of the stability of stored prothrombin-free plasmas. Using the one-stage method of Quick<sup>11</sup> the prothrombin time was first determined on freshly prepared undiluted citrated plasma. Then, 0.1 ml. of the fresh citrated plasma was diluted with 0.9 ml. of stored prothrombin-free plasma (human or bovine) and the prothrombin time determined in duplicate using the one-stage method of Quick. The prothrombin time was converted to prothrombin concentration by means of prothrombin time-concentration reference curves, and the concentration multiplied by the dilution factor of 10.

*Determination of Fibrinogen.*—The micro-Kjeldahl technique for the determination of nitrogen was employed, fibrinogen being reported in terms of clottable nitrogen. Since prothrombin was lacking in many of the plasmas a standard procedure was employed to clot fibrinogen: 15 ml. of a 1:60 dilution of a 10 per cent thrombin solution (Hemostatic Globulin)\* was added to 0.5 ml. of oxalated plasma. This mixture was allowed to coagulate for one hour at which time the fibrin was wound on a glass rod and the nitrogen content determined. Each determination was done in triplicate.

*Determination of Antithrombin.*—Imidazole buffer solution was made by dissolving 1.72 Gm. of imidazole (Eastman Kodak Company) in 90 ml. of 0.10N hydrochloric acid and diluting to 100 ml. by the addition of single distilled water.<sup>7</sup> Such a buffer has been shown to have no effect on blood coagulation.<sup>7</sup> "Buffered saline" was made by adding 10 ml. of this solution to 90 ml. of a solution of sodium chloride (85 Gm. per 100 ml.). Such buffered saline had a pH of 7.1 to 7.3. One gram of Hemostatic Globulin\* was dissolved in 100 ml. of buffered solution, and a 1:32 dilution was made with buffered saline. By adding 0.1 ml. of this 1:32 thrombin dilution to 0.2 ml. of normal plasma in a 10 by 75 mm. tube at  $37.5^{\circ}\text{C}$ ., coagulation usually occurred in twelve to thirteen seconds. An increase in antithrombin could therefore be detected by noting prolongation of the coagulation time in such a system.

*Determination of Hydrogen-Ion Concentration (pH).*—pH determinations were done using a glass electrode potentiometer (Beckman).

## RESULTS

*Stability of Prothrombin-Free Plasma (Human).*—Table I shows the effect of storage at various temperatures on the stability, thrombin time, and pH of pooled normal prothrombin-free plasma (human). Zero time in the table is taken as the completion of preparation of the prothrombin-free plasma. It is shown that the prothrombin time of fresh normal citrated plasma diluted with stored prothrombin-free normal plasma becomes progressively prolonged as the time of storage of the diluent lengthens. Such prolongation becomes manifest as early as one hour of storage at  $37.5^{\circ}\text{C}$ . and  $30^{\circ}\text{C}$ . The lower the temperature of storage the less tendency there is for such prolongation to develop over a

\*Supplied by Lederle Laboratories, Pearl River, N. Y.

TABLE I. THE EFFECT OF STORAGE OF PROTHROMBIN-FREE HUMAN PLASMA AT VARIOUS TEMPERATURES ON pH, THROMBIN TIME, AND STABILITY; CITRATED AND OXALATED HUMAN PLASMA ARE INCLUDED AS CONTROLS

[illegible]



twenty-four hour period. At  $-20^{\circ}$  C. there was no change in twenty-four hours but within ninety-six hours prolongation was manifest.\*

The duration and temperature of storage correlate well with the thrombin time in that the higher the temperature and the longer the duration of storage the greater is the antithrombic effect observed. At  $-20^{\circ}$  C. the thrombin time remained essentially unchanged over a twenty-four hour period.

Coincident with prolongation of the thrombin time at  $37.5^{\circ}$  C. and  $30^{\circ}$  C. storage, a rise in pH occurred. Although no prolongation of the thrombin time was observed in prothrombin-free plasma (human) stored at  $-20^{\circ}$  C., a rise in pH occurred in twenty-four hours that was equal to that observed at the higher temperatures of storage. The temperature of storage apparently exerted only a mild influence on the pH changes as shown in Table I. It is worthy of note that the pH changes occurred rapidly at  $37.5^{\circ}$  C. and  $30^{\circ}$  C. and then became stabilized without further rise. Similar prolongation of the thrombin time and a rise in pH to the same degree were not observed in citrated or oxalated plasma stored at  $30^{\circ}$  C. for twenty-four hours (Table I).

No significant quantitative variation in fibrinogen occurred in any of the samples during the periods of storage studied.

*Stability of Lyophilized Prothrombin-Free Normal Plasma (Human).—*In each instance the lyophilized prothrombin-free normal plasma (human) was used immediately upon reconstitution. No alteration in activity was observed when the reconstituted material was used as a diluent of fresh citrated plasma in the determination of the prothrombin time. Table II shows the numerical results of these determinations as well as the "thrombin times" and pH values done initially and after a 70-day period of storage in the lyophilized state at  $-20^{\circ}$  C. There was no observed change in the thrombin time or in the pH values. Fibrinogen determinations were not done.

*Stability of Lyophilized Prothrombin-Free Plasma (Bovine).—*Since lyophilized prothrombin-free plasma (bovine) has larger amounts of the "plasma prothrombin accelerators" resulting in acceleration of the prothrombin time,<sup>8,9</sup> it became necessary to prepare a prothrombin time-concentration curve using prothrombin-free plasma (bovine) as a diluent of

TABLE II. THE STABILITY OF STORED LYOPHILIZED PROTHROMBIN-FREE PLASMA (HUMAN) [Reconstitution with buffered distilled water was done immediately prior to each determination. The prothrombin concentration is derived from the prothrombin time-concentration curve using prothrombin-free human plasma as a diluent of fresh normal pooled human plasma (citrate).]

PERIOD OF STORAGE (WK.)	PROTHROMBIN TIME (SEC.)	PROTHROMBIN CONCENTRATION (%)	pH	THROMBIN TIME (SEC.)
0	25½	120	7.75	12
1	26	115	---	--
2	25¼	122	---	--
3	26	115	---	--
4	27	110	---	--
6	25½	120	---	--
10	26	115	7.83	11½

\*Further studies are in progress and will be reported elsewhere.

pooled fresh normal human plasma (citrate). This reference curve as well as those employing prothrombin-free plasma (human) and saline as diluents is shown in Fig. 1. The differences in the curves are considerable. It should be noted also that the prothrombin time of a 50 per cent mixture of fresh citrated normal plasma and prothrombin-free plasma (bovine) is shorter than that of the whole pooled fresh normal human plasma (citrate).

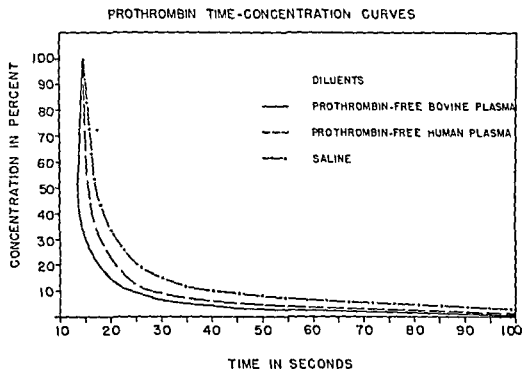


Fig. 1.

Using reconstituted lyophilized prothrombin-free plasma (bovine) as a diluent of fresh normal human plasma, no alteration in activity was observed over a 140-day period.

Thrombin times and pH values done initially and at the end of the storage period at  $-20^{\circ}\text{C}$ . in the lyophilized state showed no change in these determinations as shown in Table III.

TABLE III. STABILITY OF STORED LYOPHILIZED PROTHROMBIN-FREE PLASMA (BOVINE)  
(Reconstitution was done with buffered distilled water immediately prior to each determination. The prothrombin concentration is derived from the prothrombin time-concentration curve using prothrombin-free bovine plasma as a diluent of pooled fresh normal plasma.)

PERIOD OF STORAGE (WK.)	PROTHROMBIN TIME (SEC.)	PROTHROMBIN CONCENTRATION (%)	pH	THROMBIN TIME (SEC.)
0	21 $\frac{3}{4}$	120	7.78	11
2	21 $\frac{3}{4}$	120	---	--
4	22	117	---	--
10	21 $\frac{3}{4}$	120	---	--
20	23	110	7.80	11 $\frac{1}{2}$

#### DISCUSSION

It has been shown in the foregoing experiments that plasma rendered free of prothrombin is quite unstable when stored in the fluid state, as evidenced by prolongation of the prothrombin time of fresh plasma diluted with it; it is more

stable when frozen and maintained at  $-20^{\circ}$  C. for relatively short periods of time, but even at this temperature of storage alteration in activity is observed after several days. The prolongation of prothrombin time is not due to quantitative changes in fibrinogen. When stored in the fluid state for limited periods no change occurred in the fibrinogen content of prothrombin-free plasma and there was no apparent decrease in the fibrinogen "reactivity," as suggested by Loomis and Seegers,<sup>6</sup> since a "normal" thrombin time can be obtained by increasing the concentration of thrombin in the reaction mixture or by reducing the pH.<sup>15</sup> In addition, Alexander<sup>1</sup> has shown that the plasma of a patient with congenital afibrinogenemia exhibited prolongation of the prothrombin time similar to that of normal plasma under the same conditions of storage.

The observed alteration in stored prothrombin-free plasma (human) appears to be related in part to the development of antithrombic activity possibly resulting from treatment with barium sulfate per se, since untreated oxalated and citrated plasmas containing prothrombin do not develop antithrombin activity at the same rate of speed or to the same extent when stored at  $30^{\circ}$  C. The observed prolongation of the prothrombin time and apparent antithrombic activity have been shown to occur concomitantly with a rise in pH by Tanturi and Wetzel.<sup>15</sup> They have reported a prompt reduction in antithrombic activity in prothrombin-free plasma following the lowering of the pH to 7.6 to 7.8. In addition to this observation, the formation of fibrin by the action of thrombin on fibrinogen has been shown, by Morrison,<sup>8</sup> to be intimately concerned with the pH of the reaction mixture. Below a pII of 5.7 no fibrin will form,<sup>2</sup> while between a pH of 6 and 7 there is a noticeable increase in the rate of reaction as the pH rises. As the pII is further increased above 7, there is less obvious increase in the rate of reaction.<sup>2</sup>

From the available data reported here, it is impossible to conclude that pH changes alone are responsible for the antithrombic activity since prothrombin-free plasma (human) stored at  $-20^{\circ}$  C. in the frozen state for twenty-four hours showed an increase in pII when thawed without antithrombic activity. This is further borne out by the observations of Tanturi and Wetzel<sup>15</sup> who have shown that the antithrombic activity can be abolished without lowering the pH of the reaction mixture by the addition of protamine sulfate. They have suggested from this that stored prothrombin-free plasma may have an increased heparin content. For clarification of the relationship between pH changes and antithrombic activity further work is required.

It has been suggested<sup>15</sup> that the observed antithrombic activity of stored prothrombin-free plasma is responsible for prolongation of the prothrombin time of fresh plasma diluted with it. This would seem true only in part since other factors also are involved. If the pII of nonlyophilized stored prothrombin-free plasma (human) is adjusted to 7.4 and the plasma tested for labile factor activity (Quick),<sup>13</sup> there is found a progressive reduction in the amount of this substance as the duration of storage lengthens even though a pH of 7.4 is maintained. The lower the temperature of storage, the less tendency there is for such a reduction to occur, as noted elsewhere.<sup>12</sup> There would thus appear to be

at least two factors responsible for the prolongation of the prothrombin time of fresh normal plasma (citrate) diluted with stored nonlyophilized prothrombin-free plasma. Initially an apparent antithrombin develops causing mild delay in the speed of coagulation. After a relatively short period of storage at 37.5° C. and 30° C., there occurs gradual inactivation of the "prothrombin accelerators",<sup>9, 10</sup> which results in further slowing of the speed of coagulation. The additive effect of these two changes presumably results in a significant prolongation of the coagulation time to a limit, after which time the antithrombic effect becomes stabilized, as does the pH. The "prothrombin accelerators" finally become completely inactivated.

By lyophilizing prothrombin-free normal plasma (human) as outlined by Flosdorf and Mudd,<sup>5</sup> observing their precautions to establish the proper relationship between total volume of material and available surface during the process so as to insure proper lyophilization, it is possible to maintain such material in stable form for many weeks. By reconstituting it to a pII of 7.3 to 7.4, a diluent is obtained that is quite active and which possesses no antithrombic activity or additional loss of "prothrombin accelerators" when used promptly. Other observers<sup>11</sup> have reported increases in pII and antithrombic activity to occur in lyophilized prothrombin-free plasma. Such findings have not been observed here over a period of storage of ten to twenty weeks. Why this discrepancy exists is unknown since we have observed the same results of stability when using unbuffered distilled water for reconstitution of the lyophilized prothrombin-free plasma. It is possible that the cause of such altered activity in the material studied by others<sup>12</sup> is incomplete lyophilization.

Using barium sulfate as an adsorbing agent of prothrombin, there also occurs a reduction in certain plasma components that are intimately concerned with the speed of the coagulation reaction. To what extent barium sulfate removes the "accelerator substances" is unknown, but it is reported that approximately 50 per cent of Owren's Factor V is removed although the amount of BaSO<sub>4</sub> required to effect this removal is not stated.<sup>10</sup> The difficulty of simultaneously depleting plasma of its prothrombin and certain of the "accelerator substances" can be eliminated by using as a diluent bovine plasma treated with barium sulfate. Owren<sup>10</sup> and Seegers<sup>9</sup> both have shown that bovine plasma contains much greater quantities of their factors than does human plasma. Because of this observation, Owren<sup>10</sup> advocates the use of prothrombin-free bovine plasma as a diluent of human plasma so as to insure an excess of Factor V. Since it appears likely<sup>3</sup> that Factor V of Owren,<sup>10</sup> plasma accelerator globulin of Seegers,<sup>10</sup> and labile factor of Quick<sup>13</sup> are the same activities, it is possible to have excesses of these activities as well as an excess of plasma thromboplastin and fibrinogen in the dilution method of Rosenfield and Tuft for the determination of prothrombin concentration by using prothrombin-free bovine plasma as a diluent of fresh citrated human plasma.

In the lyophilized state prothrombin-free bovine plasma maintains unaltered activity as a reconstituted diluent of fresh human citrated plasma, and no antithrombic activity or pII changes were observed over a storage period of twenty

weeks. Because of the greater "prothrombin accelerator" activity in bovine plasma, it is necessary to establish a prothrombin time-concentration curve using prothrombin-free bovine plasma as a diluent. The shorter prothrombin time obtained at a 50 per cent concentration of prothrombin (see Fig. 1) using prothrombin-free bovine plasma as a diluent is most likely due to the greater "prothrombin accelerator" activity of this diluent.

By lyophilization of prothrombin-free normal human or bovine plasma, there is thus placed within easy reach of many laboratories and physicians a reagent whereby more accurate determinations of prothrombin can be accomplished. With proper reconstitution of a stable (lyophilized) thromboplastin and a stable (lyophilized) prothrombin-free diluent of fresh normal citrated plasma, prothrombin determinations can be performed reproducibly.

#### SUMMARY AND CONCLUSIONS

Experimental data are presented showing the great instability of normal human plasma rendered prothrombin-free by treatment with barium sulfate and stored at various temperatures in the liquid state. Such instability is shown to result in prolongation of the prothrombin time of mixtures of fresh normal citrated plasma and stored prothrombin-free plasma. This instability is thought to be due to the occurrence at 37.5° C. and 30° C. of antithrombic activity as well as inactivation of the "prothrombin accelerators." The mechanism of development of antithrombic activity is not well understood. Lyophilized prothrombin-free human and bovine plasma stored at -20° C. for ten and twenty weeks respectively are shown to possess unaltered activity as diluents when reconstituted with buffered distilled water and used immediately. A discussion is given of the advantages of using a dilution method for the determination of prothrombin concentration, employing prothrombin-free bovine plasma as a diluent of fresh normal citrated plasma. Prothrombin time-concentration curves using three different diluents are included and the need for selection of a proper diluent is obvious. It is recommended that lyophilized prothrombin-free bovine plasma, reconstituted with buffered distilled water, be used as the diluent of fresh normal citrated plasma in the determination of prothrombin concentration, as a means of supplying an excess of certain "prothrombin accelerators" to a coagulation system in which prothrombin thus remains as the only variable.

It is concluded that prothrombin-free human and bovine plasma (1) maintain stability when properly lyophilized and stored at -20° C. for ten and twenty weeks respectively, and (2) when reconstituted to pH 7.3 are ideal diluents of fresh plasma for the determination of prothrombin concentration.

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## BIOLOGIC STUDIES WITH ARSENIC<sup>76</sup>

### III. THE EFFECT OF ARSENIC<sup>76</sup> UPON THE CLINICAL COURSE OF PATIENTS WITH TUMORS OF THE HEMATOPOIETIC TISSUES

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#### INTRODUCTION

SINCE both stable arsenic and irradiation have been used independently in the treatment of tumors of the hematopoietic organs it was thought that it might be advantageous to utilize radioarsenic ( $\text{As}^{76}$ ), a substance which is metabolized like stable arsenic, exerts a similar chemotherapeutic effect, and is at the same time a source of irradiation.

$\text{As}^{76}$ , which has a half-life of 26.8 hours and which emits energetic beta and gamma rays, was prepared by pile irradiation of arsenic trioxide, and later in higher specific activity by pile irradiation of cacodylic acid. It was injected intravenously as sodium arsenite. The stable arsenic content usually varied from 3 to 10 mg. of arsenic per single injection of 1 to 80 mc. of  $\text{As}^{76}$ . Cacodylic acid, as obtained after pile irradiation at the Argonne National Laboratory, is prepared for injection by remote control from behind lead shields because of its penetrating gamma radiations. The details of the preparation of  $\text{As}^{76}$  and its metabolism have been given in two preceding reports.<sup>1, 2</sup>

Arsenic is distributed rapidly to all tissues after intravenous injection. Highest levels are reached within twelve to twenty-four hours in the spleen, liver, and kidney. Fifty per cent is excreted within seventy-two hours, primarily by the kidney. It is estimated that 1 mc. of  $\text{As}^{76}$  will deliver about 1 r. of total body irradiation.

#### METHODS

Twenty-four patients with tumors of the hematopoietic tissues, two with polycythemia rubra vera, and one with a metastatic carcinoma form the basis of this study. The diagnosis was substantiated microscopically in every case. Many of the patients had repeated biopsies of bone marrow, liver, lymph node, and spleen, which will be made the subject of a future report. (Table I.)

The first few patients in this study were treated with extremely small amounts of  $\text{As}^{76}$  as far as specific radiation effect was concerned, although receiving at the same time enough stable arsenic probably to exert a mild chemotherapeutic effect. The first doses attempted were about 0.5 to 2.0 mc., but within a short period of time this was increased, partly because of experience with the inadequacy of these doses and partly because of the availability of arsenic with a higher specific radioactivity. As much as 90 mc. with about 5 mg. of stable arsenic were administered in a single injection to the recently treated patients. In addition, each patient received such supplemental treatment as transfusions, antibiotics, Digilanid and mercurials, and toluidine blue or protamine sulfate as indicated.

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TABLE I. DIAGNOSIS, DOSAGE, AND RESPONSE TO TREATMENT WITH As<sup>76</sup>

DIAGNOSIS	CASE	MILLICURIES OF As <sup>76</sup>	RESULTS
Acute leucemia	1	24 on 10/24/47 60 on 11/12/47	No remission, died of hemorrhage
	2	69	No remission, died of hemorrhage
Subacute myelogenous leukemia	3	13.5 on 10/3/47 12 on 10/10/47 45 on 1/31/48 47 on 5/14/48	Three remissions of 2 to 3 mo. each.
	4	17	Inadequate dose, died of intracranial hemorrhage
	5	90	Remission of 1 wk., then steady downhill course.
	6	30	Symptomatic improvement for 2 wk. until death from cerebral hemorrhage
Chronic myelogenous leukemia	7	30 on 3/31/47 54 on 2/1/49	First remission of 5 mo., second of 2 mo.
	8	60	Good remission for 4 mo.
	9	40	Good remission for 19 mo.
	10	32 on 6/11/48	Six-week remission on inadequate dose, second remission of 2 mo.
	11	59 on 1/28/49 69 on 2/4/49 36 on 2/11/49	Symptomatic improvement 1 mo.
	12	36 on 1/30/48	Little improvement, concurrent hemolytic anemia
Subacute lymphatic leukemia	13	39 on 2/6/48 60	Remission of 2½ mo. but had series of pyogenic abscesses
	14*	14	Remission of 1 mo., then died of hemorrhage
	15	72	Remission of 1 mo., then died of generalized tuberculosis
Chronic lymphatic leukemia	16	38	No response, in severe mental depression at time of treatment
	17	38	Died of cerebral hemorrhage after 4 wk. of symptomatic improvement
	18	80	Two-week remission, died of pericardial effusion
	19	76	Five-month remission until death from congestive heart disease
Polycythemia rubra vera	20	45	Three-month remission
Multiple myeloma	21	15 on 8/28/47 12 on 9/5/47	Six-month remission (unusually benign type of case)
	22	88	Seven-month remission
Mycosis fungoides	23	80 on 9/10/48 80 on 9/24/48	No response, later responded to x-ray
Metastatic carcinoma, primary in stomach	24	80	Decrease in size of liver but progressive downhill course

\*Referred by Dr. Arvid Johnston, Rockford, Ill.

## RESULTS

*Hodgkin's Disease.*—Each of four patients with Hodgkin's disease received from 1.0 to 13.0 mc. of As<sup>76</sup> and up to 52 mg. of stable arsenic in divided doses. The longest remission, two months, was obtained in a patient receiving 52 mg. of stable arsenic and 13.6 mc. of As<sup>76</sup>. However, it was felt that the amount of stable arsenic was such as to be sufficient in itself to produce a remission, and that the As<sup>76</sup> had not been given an adequate trial in this group.



*Acute Leucemia.*—Both patients experienced a sharp drop in the white count and platelets but no significant change in the differential count (Fig. 1). Case 1 developed a severe hemorrhagic process and Case 2 had a terminal rise in the white count to 180,000. Both patients had an intractable anemia which failed to respond to therapy of any nature including massive transfusions.

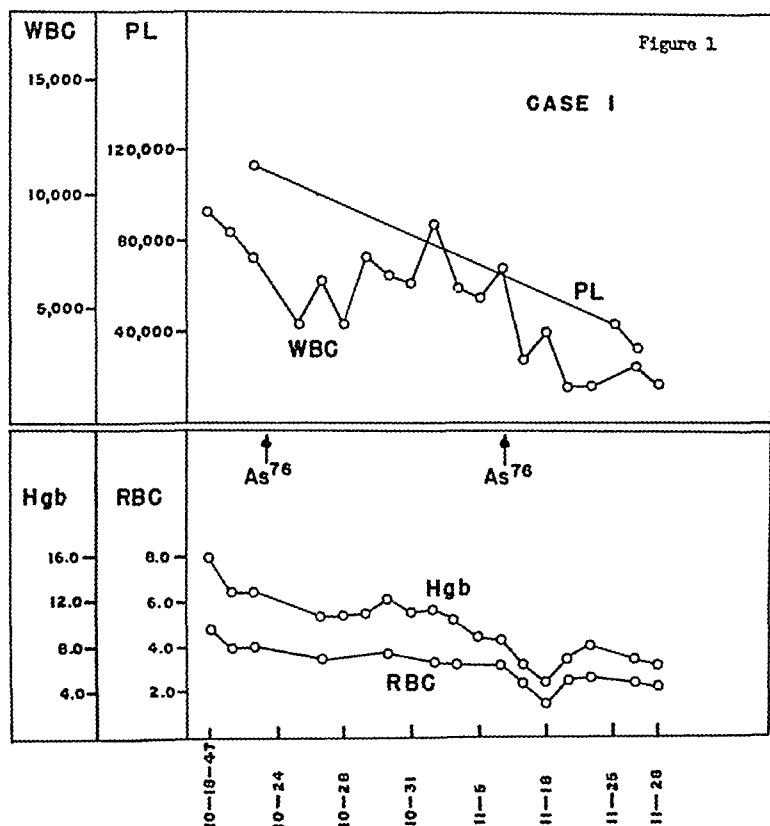


Fig. 1.

It is clear that these two patients failed to show any better response to  $As^{76}$  than has been noted in the past to urethane,<sup>3</sup>  $P^{32}$ ,<sup>4, 5</sup> nitrogen mustard,<sup>6-9</sup> or x-ray.<sup>10</sup> It is probable that Aminopterin<sup>11, 12</sup> will have more to offer than  $As^{76}$  in the treatment of acute leucemia.

*Subacute Myelogenous Leucemia.*—Case 4 did not receive enough  $As^{76}$  to exert a therapeutic effect. Case 5 had an extremely short remission but the patient was in very poor condition when treatment was begun. If the result obtained in Case 3, in which there were three remissions varying from about four months to two months in spite of an intractable osteomyelitis, can be duplicated in other cases of subacute myelogenous leucemia, it would appear that  $As^{76}$  may be about as effective as Aminopterin<sup>12</sup> and superior to the more common therapeutic agents<sup>4, 7-10, 13</sup> and radiosodium<sup>14</sup> in the treatment of this condition.

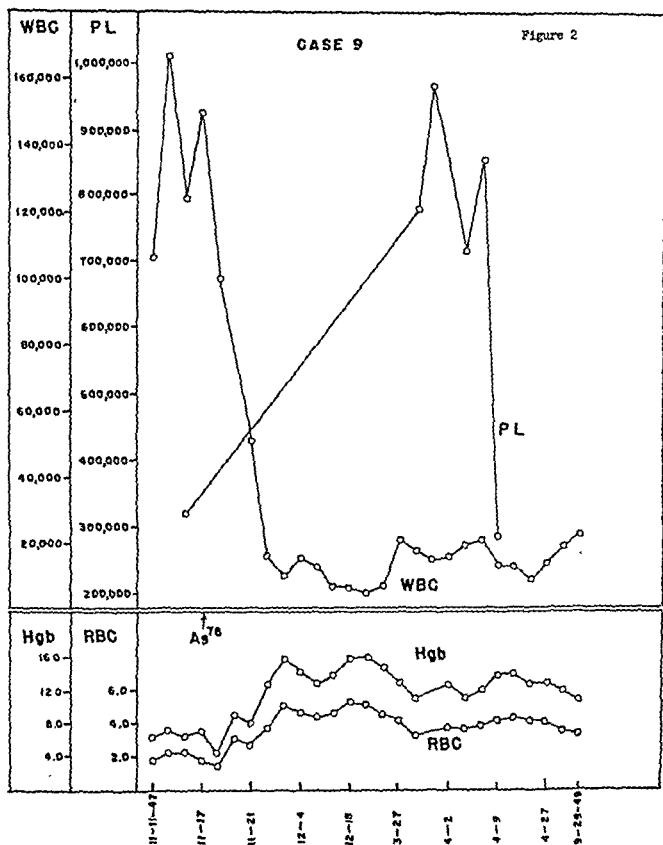


Fig. 2.

*Chronic Myelogenous Leucemia.*—Four (Cases 6, 8, 9, and 11) of the six patients were previously untreated and one (Case 7) had one course of urethane and should also be classified as an early case. Four patients responded with remissions from four to sixteen months in duration (Fig. 2) and two (Cases 7 and 10) responded in a similar manner to a second course of As<sup>76</sup>. The other untreated patient (Case 11) had only slight symptomatic improvement. The sixth patient (Case 6), refractory to x-ray therapy, died

of a cerebrovascular accident. However, his anemia failed to respond to transfusions following  $\text{As}^{76}$  and he may be classified as a therapeutic failure as is usually the result in patients of this type.<sup>4</sup>

Chronic myelogenous leucemia is a disease with great variations in its course. Patients have been known not to develop any subjective symptoms for as long as five years after the diagnosis was established.<sup>15, 16</sup> However, only a minority (16 per cent) of patients have spontaneous remissions once the disease produces anemia or subjective symptoms.<sup>15</sup> Therefore, any good result obtained in patients who were treated merely on the basis of a positive diagnosis may be ascribed to the benign nature of the disease rather than to the direct effect of therapy.

With this reservation in mind it would appear that even the greater differences in response reported in previously untreated patients following x-ray,<sup>16-18</sup>  $\text{P}^{32}$ ,<sup>4, 5, 18</sup> urethane,<sup>3, 19</sup> Fowler's solution,<sup>20</sup> and  $\text{Na}^{24}$ <sup>14</sup> may well be due to variation in the biologic nature of each patient's disease. It would, therefore, appear that remissions of four to sixteen months in our untreated cases represent no better than an average response (Fig. 2). Like other generalized methods of therapy,  $\text{As}^{76}$  is not as efficacious in reducing the size of a huge spleen as local x-ray therapy,<sup>4</sup> but like  $\text{P}^{32}$ <sup>4, 5, 18</sup> and  $\text{Na}^{24}$ <sup>14</sup> it rarely produces any toxic symptoms.

*Subacute Lymphatic Leucemia.*—Two of the four patients (Cases 13 and 14) were previously untreated and had symptoms of comparatively short duration. One patient, Case 15, had received x-ray therapy with very little benefit and one, Case 12, had received urethane without improvement. Three (Cases 13, 14, and 15) responded to therapy with an improvement in appetite, decrease or absence of fever, decrease in lymphadenopathy, and lowering of the white count for about two months. However, two (Case 13 and 15) of these three experienced the onset of a severe infection, furunculosis in one, tuberculosis in the other.

The fourth patient (Case 12) had a hematologic response with a drop in white count and a decrease in fever, but because of an associated hemolytic anemia he continued as an invalid until his death one year later following splenectomy in another institution.

These results are, in general, comparable with those reported in the literature following Aminopterin.<sup>12</sup> Possibly subacute lymphatic leucemia may show a better response to  $\text{As}^{76}$  than that reported after  $\text{P}^{32}$ ,<sup>4, 5</sup> urethane,<sup>3, 19</sup> or nitrogen mustard.<sup>13</sup> The occurrence of two severe infections (Cases 13 and 15) at the time of greatest depression of the white count should warn against the use of  $\text{As}^{76}$  in the presence of any generalized infection, especially tuberculosis. We have been impressed by the occurrence of a flare-up in cases of previously quiescent tuberculosis following generalized cytotoxic therapy such as  $\text{As}^{76}$  and nitrogen mustard in the treatment of leucemias and lymphomas.<sup>21</sup>

*Chronic Lymphatic Leucemia.*—Four patients were treated with fairly adequate amounts of  $\text{As}^{76}$ . Only one (Case 16) was previously untreated. He was in a severe mental depression and failed to respond to therapy. Two patients (Cases 17 and 18), each of whom had been subjected to extensive x-ray

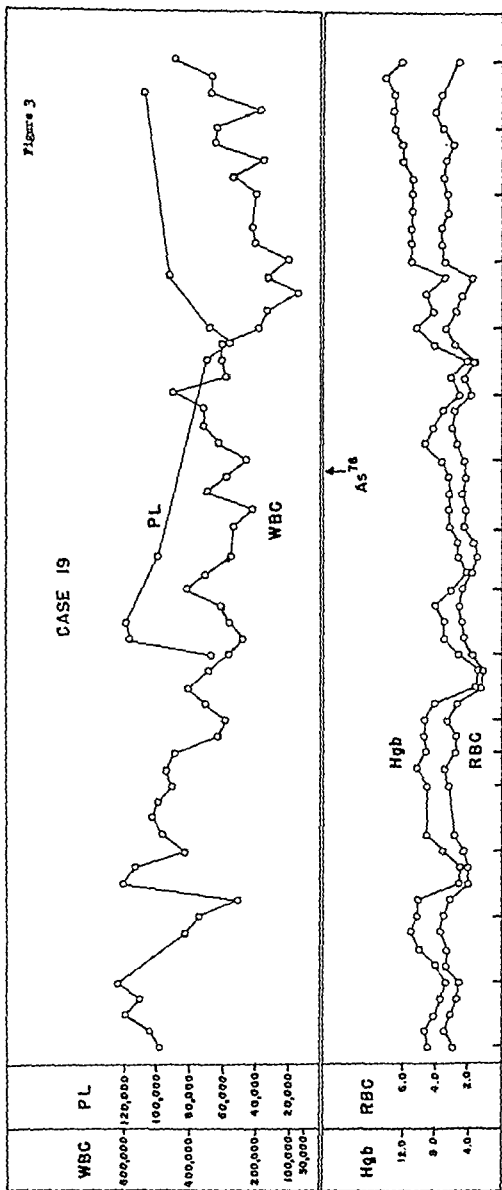


Fig. 3.

therapy in the past, succumbed to complicating diseases. A spectacular result was obtained in the last patient (Case 19) since an intractable anemia was relieved and massive lymphadenopathy and splenomegaly were decreased (Fig. 3).

Our results, in general, are not as favorable as those reported after x-ray<sup>16, 22</sup> or P<sup>32</sup>,<sup>4</sup> nitrogen mustard,<sup>7</sup> urethane<sup>3, 19</sup> or Na<sup>24</sup>.<sup>14</sup> Particularly discouraging was the complete failure in the patient, Case 16, who was previously untreated. However, Reinhard<sup>4</sup> has encountered a similar result after P<sup>32</sup>, as we have after nitrogen mustard.<sup>21</sup> The response of Case 19, who had remained severely anemic in spite of massive transfusions and several types of therapy, was most encouraging since this is the type of case which is notoriously difficult to manage.

*Polycythemia Rubra Vera.*—The response of Case 20 was fairly satisfactory and also was qualitatively similar to that described after P<sup>32</sup>.<sup>4, 5, 23</sup> However, much longer remissions are not at all unusual after P<sup>32</sup> so that As<sup>76</sup> has little to offer that is superior to the results already achieved by P<sup>32</sup>. Another patient was treated with a total of 2.4 mc. of As<sup>76</sup> and phlebotomies. Since this amount of As<sup>76</sup> is too small to be effective, the brief remission probably was due to the phlebotomies.

*Multiple Myeloma.*—This disease is almost always characterized by a rapidly progressive downhill course with unrelenting pain in untreated cases, although occasional chronic cases such as Case 21 have been recognized.<sup>24</sup> Remissions similar in extent to that experienced in Case 22 have been reported after urethane<sup>25</sup> and Stilbamidine.<sup>26, 27</sup>

*Mycosis Fungoides.*—In many respects the therapeutic results in this disease are similar to those achieved in Hodgkin's disease. In Case 23, treated four months before death, it was quite clear that the skin and pulmonary lesions were still in part amenable to local x-ray therapy after they had failed to respond to As<sup>76</sup> or nitrogen mustard.

*Metastatic Carcinoma.*—The results in Case 24, in which the patient had a carcinoma of the stomach, are difficult to interpret. Carcinoma of the stomach may occasionally give rise to masses that respond to x-ray therapy by a decrease in size as did this patient's liver metastases. There was little else in the patient's course to suggest that As<sup>76</sup> would prove curative or even beneficial in carcinoma of the stomach.

#### DISCUSSION

It appears, therefore, that As<sup>76</sup> resembles numerous other therapeutic agents such as x-ray, urethane, nitrogen mustard, radiophosphorus, radio-sodium, and Fowler's solution in that it is capable of producing a remission in certain of the more chronic and benign tumors of the hematopoietic tissues and that it is not a cure for these diseases. The more acute forms of these diseases, with the possible exception of subacute leucemia, do not seem particularly amenable to As<sup>76</sup> therapy.

In evaluating the clinical usefulness of As<sup>76</sup> one is forced to consider whether it is more effective than previously used materials in producing a re-

mission, whether it will produce a remission where other agents have failed, and whether the advantages, if any, are sufficiently great to warrant using a substance that is so difficult and expensive to prepare and so dangerous to handle. Unfortunately, because of numerous factors, such as intercurrent disease, variations in the clinical course of patients with the same disease, duration of disease, previous therapy, supplemental therapy, and rest and psychic factors, it is very difficult to compare any series of patients. One cannot but be impressed by the occasional patient who responds to therapy in a manner that is entirely unexpected and inconsistent with the usual response characteristic of patients with that disease.

The criteria for evaluating a clinical remission are not similar. For example, depression of the white count has frequently been used as a means of estimating the response to therapy. However, particularly in acute leucemia and the terminal stages of chronic leucemia, the height of the white count may bear no relation to such response. In this study we have followed the blood counts carefully and have noted a depression of the white count varying from 20 per cent to 80 per cent of the pretreatment figure in almost every patient treated without an invariable improvement in the clinical condition of the patient. Consequently the white counts have not been reported in detail. There has been no marked tendency of the peripheral blood differential count to return to normal following treatment except in a few cases of chronic myelogenous leucemia. An alleviation of the anemia is generally acceptable as an indication of a good therapeutic response. Since practically every one of the patients in this study received numerous transfusions, this criterion was rendered unreliable.

In general, we have felt that the ability of the patient to live a more comfortable and more useful life was the best indication of a response to therapy.

On this basis our results seem to have been unusually good in a single case of chronic lymphatic leucemia characterized by severe anemia, possibly favorable in subacute lymphatic leucemia, and worthy of further evaluation in multiple myeloma. In the other diseases treated the recognized agents such as x-ray, P<sup>32</sup>, urethane, and nitrogen mustard, which are more readily available, less expensive, and less hazardous, will give similar if not superior clinical results.

It is of importance to note that we have not yet attained a maximum safe clinical dosage level of As<sup>76</sup> nor have we fully explored the combination of stable and radioarsenic or the effect of repeated small doses of As<sup>76</sup>. It is possible that when these are attained a better clinical response may be achieved. However, the problems of handling the amounts of As<sup>76</sup> we have used are so great that it hardly seems practical or worth while to attempt to use larger quantities of As<sup>76</sup> on the basis of the response of the patients in this study.

#### CONCLUSIONS

1. Twenty-four patients with tumors of the hematopoietic tissues, two with polycythemia rubra vera, and one with metastatic carcinoma have been treated with intravenous injections of radioarsenic (As<sup>76</sup>).

2. This radioisotope, under the conditions used in this study, appears to offer no advantages over the types of therapy already in use for these conditions.

We wish to thank Mr. Howard Ducoff and Mr. Robert L. Straube of the Argonne National Laboratory for their cooperation in the preparation of the  $As^{76}$  used in this study.

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# THE USE OF RADIOACTIVE SILVER FOR THE DETECTION OF ABSCESSES AND TUMORS

## I. THE CONCENTRATION OF $\text{Ag}^{111}$ IN SPONTANEOUS AND EXPERIMENTALLY INDUCED ABSCESSES

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**I**N A recent communication from this laboratory<sup>1</sup> it was pointed out that tracer doses of the long half-life silver isotope,  $\text{Ag}^{108, 110}$  (half-life 225 days) could be caused to concentrate at particular localities in the bodies of albino rats by the simple expedient of inducing infection (experimentally) in the form of abscesses. It also appeared that the radioactive isotope concentrated itself in areas of spontaneous infection. It seems possible that if the short half-life radio-silver isotope,  $\text{Ag}^{111}$  (half-life 7.5 days) could be concentrated at or near the site of a lesion in sufficient amounts it might prove of value in therapy of tumors in human beings.

It is clear that if radiosilver can be caused to concentrate in abscesses induced by injection of a suspension of bacteria, or in areas of spontaneous infection, the procedure may become a very valuable method for the detection or location of hidden or obscure abscesses. The existence of the 8.2 day half-life  $\text{Ag}^{106}$  and the newly developed, highly sensitive, directional Geiger-Müller counters make the procedure ideal as a tool for diagnosis in the patient with an obscure focus of infection.\*

In the part which follows it will be shown that the  $\text{Ag}^{111}$  behaves similarly to the  $\text{Ag}^{108, 110}$  (as would be expected from the electron configurations), viz., it concentrates in spontaneously or experimentally induced infected areas. The directional Geiger-Müller counter was not used in these experiments with  $\text{Ag}^{111}$ . It was necessary to conduct the assay through actual ashing of the tissues and chemical isolation of the radiosilver.

### EXPERIMENTAL

The  $\text{Ag}^{111}$  was separated from the irradiated palladium target obtained from the pile at Oak Ridge by a procedure developed in the Cancer Research Laboratory of this Institution by Rouser and Hahn.<sup>2</sup>

The test organism used was a strain of *Streptococcus hemolyticus* isolated from the throat of a hospital patient. The organism was used after sixteen hours of incubation in proteose peptone broth at 37° C. One tenth of a milliliter of the undiluted culture was injected into the right legs of albino rats and forty-eight hours later 0.5 milliliter of

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\*The  $\text{Ag}^{106}$  isotope is the isotope of choice for this purpose since it produces gamma rays and, unlike  $\text{Ag}^{111}$  which is a pure  $\beta$ -ray emitter, should be amenable to external survey. Pure  $\beta$ -ray emitters are useless for this purpose since their particles travel only short distances. The 225-day half-life isotope,  $\text{Ag}^{108, 110}$ , while it emits gamma rays and thus would be detectable in external surveys, is excluded for this purpose on account of the danger of prolonged irradiation resulting from its long half-life. This would be particularly hazardous if for some reason the isotope is not promptly excreted. On the other hand,  $\text{Ag}^{106}$  has a relatively short half-life of 8.2 days.

$\text{Ag}^{111}$  in the form of the nitrate was injected either into the same leg or into the left one. Three days after injection of the isotope the animals were sacrificed and the legs, kidneys, livers, lungs, hearts, and testes or ovaries were ashed and the silver was isolated and subjected to radioassay as described previously.<sup>1</sup> The results with five animals are given in Table I. Each tissue was subjected to bacteriologic examination before assay.

TABLE I. DISTRIBUTION OF  $\text{Ag}^{111}$  IN VARIOUS TISSUES 72 HOURS FOLLOWING ITS INJECTION IN LEFT OR RIGHT LEG IN THE PRESENCE OR ABSENCE OF INFECTION EXPERIMENTALLY INDUCED IN THE LEFT LEG  
(DOSE: 0.5 C.C. WITH  $3.62 \times 10^4$  COUNTS PER MINUTE)

RAT	RIGHT LEG	LEFT LEG	KIDNEY	LIVER	LUNG	HEART	TESTES OR OVARY	RIGHT LEG	LEFT LEG
1		Isotope	310	820	85	55	780*	1,220	2,620
		Organism							
2		Isotope	400	862	75	40	18	1,065	3,220
		Organism							
3	Isotope	Organism	492	1,690*	91	80	20	1,620	4,000
4	Isotope	Organism	472	1,261	97	49	27	1,026	3,221
5		Isotope	472	1,097	101	52	23	1,262	1,415

\*Bacteriologic test positive, indicating infection.

It was of interest that the concentrations of radiosilver were uniformly high in livers. To confirm this observation, three additional animals were given injections of  $\text{Ag}^{111}$  alone and the concentrations of the isotope in kidney, liver, lung, heart, and testes or ovaries were determined. The results are shown in Table II where it is to be seen again that the livers have a uniformly high count.

TABLE II. DISTRIBUTION OF  $\text{Ag}^{111}$  IN VARIOUS TISSUES 72 HOURS AFTER ITS INJECTION INTO RIGHT LEG

RAT	RADIOSILVER INJECTED INTO RIGHT LEG (COUNTS PER MIN.)				
	KIDNEY	LIVER	LUNG	HEART	TESTES OR OVARY
1	388	1,031	41	0	0
2	381	924	51	0	0
3	403	964	55	0	0
Control sample	36,147				

An additional three animals were studied for normal distribution of the isotope. In this instance the studies were extended to include as much of the muscle, bone, and blood as could be obtained and the feces and urine were collected. The stomachs and intestines were assayed together as well as the major portion of the skin. The heads and tails were not studied. The results are shown in Table III. Rat 1 must not be compared with Rats 2 and 3 since a massive infected area was found in the left leg (right leg injected with  $\text{Ag}^{111}$ ). The predominating organism in this infected organ was a strain of *Str. hemolyticus*. The tissue placed under the Geiger-Müller tube for radioassay was found to be highly radioactive.

#### DISCUSSION OF RESULTS

The experimental findings given in Table I indicate, in line with our previous results with the 225-day half-life isotope, that  $\text{Ag}^{111}$  also concentrates in the tissue with the experimentally induced abscess. They demonstrate also that this will occur even if the isotope is injected into an organ quite remote from the focus of infection. The result with Rat 1, Table III, was extremely fortunate, though unexpected. The radiosilver was injected into the right leg. When the

TABLE III. DISTRIBUTION OF  $\text{Ag}^{111}$  IN TISSUES OF THE ANIMAL BODY AND RECOVERY AFTER 72 HOURS  
(HEADS AND TAILS NOT STUDIED. RAT 1 WAS FOUND TO HAVE A MASSIVE INFECTED AREA IN THE LEFT LEG)

TISSUE	RAT 1 (COUNTS/MIN.)	RAT 2 (COUNTS/MIN.)	RAT 3 (COUNTS/MIN.)
Liver	65	72	82
Lungs	11		
Kidney	26		
Skin		3	11
Bone		10	7
Muscle	5,179	122	130
Feces	879	5,763	5,970
Urine			
Blood	39	22	30
Testes or ovaries			
Intestine and stomach	29	114	122
Heart		35	21
Per cent recovery	78.03	85.14	88.36
Control sample (counts/min.)	7,981	7,212	7,212

animal was sacrificed a massive infection of the left leg was discovered. The high count in the muscle fraction for this animal was chiefly due to the silver concentrated in this area. This result emphasizes the probable value of the method, if employed, for detection of obscure or hidden abscesses.

The uniformly relatively high count in the livers of the animals studied deserves some comment. The counts in the infected tissue are some three to four times as great as those seen in the livers and thus would hardly be cause for confusion in the search for a focus of infection. These relatively high counts after three days are apparently related to excretion of the silver. The high counts seen in the feces, the absence of silver from the urine, and the relatively high values for liver lead to the conclusion that tracer doses of radiosilver are excreted by the liver, presumably by way of the bile, into the intestines and excreted in the feces. These results confirm those of Scott<sup>3</sup> recently available from the Atomic Energy Commission.

Previous attempts to develop procedures for the detection of abscesses have been made by Kroll, Strauss, and Neeheles<sup>5, 6</sup> and Strauss, Neuwelt, Rovner, and Neeheles.<sup>4</sup> In a recent publication<sup>6</sup> these authors suggest the use of the bis-azo dye, disodium-1-amino-8-hydroxy-naphthalene-3,6-disulfonate (H-acid) monobrominated with radioactive bromine for localization in experimentally induced abscesses in dogs. The preliminary experimental results reported here would suggest that the employment of radiosilver nitrate in tracer doses may be a more simple procedure.

These studies are being extended to animals with the turpentine sterile abscess and with tumors.

#### CONCLUSIONS

1. The radioactive  $\text{Ag}^{111}$  isotope concentrates in areas of infection.
2. This isotope is excreted by the liver into the intestine presumably by way of the bile and the feces.

3. Since both  $\text{Ag}^{108, 110}$  and  $\text{Ag}^{111}$  concentrate in areas of experimentally induced and spontaneous infections, it is to be expected that other silver isotopes such as  $\text{Ag}^{106}$  for example, would behave similarly. Since  $\text{Ag}^{106}$  has a short half-life (8.2 days) and also emits gamma rays and is thus amenable to external survey, it is suggested that it might prove to be of value as a tool for the detection of obscure foci of infection in the animal body.

The senior author wishes to express his gratitude to Dr. P. F. Hahn of the Cancer Research Laboratories of this College for his assistance in the initiation of the program of isotope research.

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# THE MINIMAL SODIUM DIET: A CONTROLLED STUDY OF ITS EFFECT UPON THE BLOOD PRESSURE OF AMBULATORY HYPERTENSIVE SUBJECTS

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## INTRODUCTION

DESPITE the extensive studies on the place of diet low in sodium chloride in the therapy of hypertension,<sup>1-5</sup> there is as yet no complete agreement as to the efficacy or explanation of this therapy. Recent re-examination of this subject might lead one to the conclusion that the administration of diets which are low in sodium is sometimes attended by a reduction in blood pressure.<sup>6, 7, 8</sup> This, it has been claimed,<sup>6, 9</sup> is a reason for the success<sup>10</sup> of the rice diet therapy. Dietary restriction of sodium (including the rice diet) has again become widespread and is generally being applied in clinical practice without adequate control. Several carefully controlled studies have dealt with patients in the hospital after they have reached a stabilized, or nearly stabilized, blood pressure level and in whom a period of sodium restriction has then been alternated with a period of more normal sodium intake.<sup>11, 12, 13</sup> From these studies has come the observation that in some patients a fall in blood pressure may occur during a period of severe sodium restriction, but that this fall may be slight and may occur only when the twenty-four hour intake of sodium is kept extremely low.

It is not satisfactory to carry over the results of these studies by attempting to apply them generally to the clinical treatment of hypertension. First of all, a hospital environment is not the usual environment for a human being. The factors which may relate to the maintenance (and perhaps the establishment) of hypertension are considerably modified by the simple fact of being in a hospital as a patient. Therefore, a form of therapy which is ineffective or relatively ineffective in the hospital might conceivably be more effective when the patient is under "normal" living conditions.

Second, it is possible that severe sodium restriction might serve to reduce blood pressure in the hypertensive patient only under certain circumstances. For instance, sodium restriction may be unable to further lower a blood pressure that has come down under influence of other factors to a minimum or "base line." If the patient had been treated at a higher level of blood pressure than this "base line," an effect might have been noted, even though the therapy is ineffective in lowering the "floor" of blood pressure.

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Most important of all, the administration of a diet which is rigidly restricted in any factor may influence the habits and reactions of the patient quite profoundly. In this manner it may produce physiologic changes unrelated to the removal of the particular agent under study. In order to control this most prominent feature of dietotherapy it is mandatory to administer a diet identical to the experimental diet in every way, except for the factor under study. Moreover, this must be done in such a way that the subject remains unaware of the difference. In addition, in any study where the interests, emphasis, or bias of the investigator might modify the results, he, too, should not know whether his observations relate to the experimental or the control period.

Two recent studies have been reported on the effect of rigid sodium restriction upon ambulatory patients in which favorable results are claimed.<sup>7, 8</sup> These reports do not include controls, either of the diet in terms of the degree to which the diet was followed or of the factors in the treatment, other than lack of sodium, which might be responsible for the results.

#### PLAN OF STUDY

For these reasons a controlled study was planned in which all patients would be placed upon the same dietary regimen—one which would be rigidly restricted in sodium content to less than 300 mg. of sodium per day. The experiment was divided into three periods of six weeks each. Schedules were prepared for twenty-four patients covering the administration of either sodium chloride,\* 4 Gm. per day, or an identically designed lactose preparation\* to these patients during the three experimental periods. (A construction based upon three periods was chosen to make it impossible to be certain whether the medication was being changed or not at the conclusion of any one period.) Of the eight different combinations of periods possible, these four were selected: 1. Lactose, sodium, lactose; 2. sodium, lactose, lactose; 3. lactose, lactose, sodium; 4. sodium, lactose, sodium.

A schedule was selected in random fashion by the pharmacist† and assigned to each patient, the medication to be issued from the pharmacy according to this schedule and without knowledge of the patient or the clinic personnel. Although occasionally a patient had gastrointestinal distress which might suggest that he was receiving sodium chloride, in the main there was no indication as to which medication was being taken.

The subject was seen throughout by the same investigator, who, following a uniform technique each week, recorded the blood pressure on one particular arm after the patient had rested in the recumbent position for at least five minutes, averaging two or three determinations. The recommended technique for blood pressure measurement was followed.<sup>16</sup>

Adhering to the diet as planned was not an easy task. As a practical check, twenty-four hour urine samples were collected usually once a week for sodium analysis, with creatinine analysis to establish the completeness of the sample. While urinary sodium will not parallel dietary sodium during retention, in diuresis or other state of negative sodium balance, the length of the observational periods makes it unlikely that any significant storage or loss could occur and not be reflected by weight change.

The technician alone had access to the records of medication. Accordingly, if the sodium excretion exceeded the estimated intake, the doctor and dietician could be so informed. The checking procedure resulted in some delay and this explains why a number of patients were permitted to continue the experiment although their sodium intake was so high that they

\*These were initially provided as gelatin capsules containing 0.5 Gm through the courtesy of Dr. L. E. Josselyn and the Abbott Laboratories, North Chicago, Ill. Subsequently, specially coated, sealed tablets were used, obtained through the courtesy of Dr. K. G. Kohlschadt and Eli Lilly & Company, Indianapolis, Ind.

†We are greatly indebted for the able cooperation of Mrs. M. L. Kettering and her staff.

cannot now be considered to have been on a rigid sodium restriction. While some patients will follow the diet with precision and have no difficulty in maintaining a urinary twenty-four hour sodium well below 300 mg., other patients cannot be made to understand, or do not want to accept, the limitation of a diet so constructed.

*Selection of Diets.*—The diets were individually prescribed after interview. They attempted to meet the doctor's prescription as to caloric level (maintenance was desired) and as to protein content (usually as high as the patient would accept), to consider the patient's tastes, and still to have the calculated sodium content below 300 milligrams. A commercial preparation of salt-depleted reconstituted milk\* was employed where indicated. Commercial salt substitutes were permitted if they contained no sodium! Iron and vitamin deficiencies were corrected by medication where necessary. Water was allowed ad libitum, the sodium content being known or determined to be insignificant. In several instances "holidays" were permitted for Christmas or New Year's Day. Although these were followed by a mercurial diuretic and when necessary an additional urinary collection, relaxation to this degree was a mistake, at least in the handling of the experiment. We did feel at the time that it kept the cooperation of the subject. We were aware of the variation in sodium content of foods according to selection and preparation, as well as differences in values cited by different authorities.<sup>17, 18</sup>

*Selection of Patients.*—†Twenty-four patients were selected on the basis of the existence of definite diastolic hypertension (above 90 mm. Hg) after several Clinic visits. More than half of the patients had long records of previous attendance at the Clinic. A group was chosen who expressed themselves as willing to follow the procedure with the understanding that they were participants in an experimental study. Since the experiment contained its own inherent control it was not thought necessary to observe these patients for any period on a normal diet prior to placing them on the experimental regime. In addition, a type of incomplete control was available in the records of those patients who had been attending the clinic previously, but in these cases blood pressures were not taken always in the same manner or by the same observer. A second type of control was offered in the several patients in whom diet was begun before the "special" medication was available, and these patients, therefore, had a period of sodium restriction during which time they were not given medication. In one or two instances after the experiment was completed and it was determined that the patient had not followed the diet correctly, a final experimental period was added during which no "special" medication was given and the patient was observed on the salt restriction alone. The ages of the patients were between 28 and 70. Nine were men. The etiology of hypertension was considered to be known in two cases, one of glomerulonephritis and one of pyelonephritis with nephrolithiasis. Two patients previously had undergone sympathectomy. None of the patients were in congestive heart failure, renal insufficiency, showed hypoproteinemia or lowered serum sodium or chloride concentrations, or had visible edema. Of the twenty-four subjects selected, twenty-one continued the study for at least two experimental periods and twenty of these for at least three periods. In some instances repeated determinations were made of the heart size by roentgenogram, the electrocardiogram, blood volume, and thiocyanate available space; but not enough information was obtained over the short period of this study to warrant any attempt at analysis. Other forms of medication which did not augment sodium intake (digitalis, phenobarbital, thiocyanate) were either discontinued at the outset, or continued unchanged during experimental and control periods.

## RESULTS

The entries, representing the average of the blood pressures recorded at each visit, were averaged for each experimental period. The values obtained are considered to represent the blood pressure for the subject under the conditions

\*Generous supplies of Lonalac were made available by Dr. C. E. Bills of Mead Johnson & Company, Evansville, Ind.

†Neocurtessal, Winthrop Chemical Company, Inc., New York, N. Y.; K-Salt, Chicago Dietetic Supply Co., Chicago, Ill.

‡Dr. A. S. Alving and Dr. R. M. Becker referred several of the patients used in this study.

of the experiment. (If, as has been claimed, an effect of sodium restriction may not appear for some time after the restriction of diet, and may persist after discontinuance of the regime, then the averages thus taken will diminish the apparent effect.)

As a first analysis the blood pressures were averaged for all lactose periods for each patient and compared with the blood pressure obtained during the period or periods when sodium chloride was given. This construction assumes that the experiment was carried out as it had been planned originally. An analysis of the diastolic pressures according to this grouping fails to reveal any significant differences between intended experimental and intended control periods in the group as a whole. The subjects were then reclassified according to the results of the averaged twenty-four hour urinary sodium assay. It is recognized that a single twenty-four hour urinary collection may not represent the average of the excretion of sodium for a seven-day period, but by the same criterion the observations of blood pressure made one day in the week and only during one part of the day do not necessarily represent the average blood pressure of that patient over the entire week. With this realization, the experimental periods have been arranged under three headings. Where the twenty-four hour urinary sodium samples averaged less than 0.5 Gm. during a period, the patient was considered to have been on rigid sodium restriction (A). When the average of the samples of twenty-four hour urinary sodium was greater than 0.5 Gm. but less than 1.05 Gm., the degree of sodium intake was considered to have been intermediate (B). When the twenty-four hour sodium samples averaged 1.05 grams or more, the sodium intake was not considered restricted (C). On this basis, only nine patients were observed to have actually maintained a rigid restriction for at least one complete period on an average basis. Two of these patients were on the restricted diet for two periods apiece. One patient was carried for only two periods and during both of these was on a restricted sodium intake.

There emerges, therefore, a group of eight patients who were under severe sodium restriction for at least one period and by comparison had at least one period of intermediate or relatively unrestricted intake of sodium. Table I indicates the average blood pressure during the experimental and the control periods for each of these subjects together with the experimental difference produced by the substitution of sodium chloride for lactose. The observed mean change in diastolic pressure was -4.7 mm. of mercury with a standard deviation of 7.6. The mean difference between the average of systolic pressures was -8.7 mm. of mercury with a standard deviation of 10.1

It may be argued that these differences are entirely due to chance. As a test of the adequacy of our method of sampling blood pressures, the observations on twenty-one patients used in this study may be employed. While only eight of the group had been suitable to compare in experimental and control situations, twenty-one of the subjects had been followed for at least *two like* periods. These may have been periods each of low sodium excretion, or periods



TABLE I. AVERAGES OF BLOOD PRESSURES TAKEN DURING CONTRASTING SODIUM RESTRICTION PERIODS (DURING PERIODS A THE AVERAGE SODIUM EXCRETION WAS BELOW 0.5 GM./24 HR. DURING B AND C IT WAS GREATER)

SUBJECT	SYSTOLIC (MM. HG.)			DIASTOLIC (MM. HG.)			NUMBER OF ENTRIES	
	A	B OR C	DIFF.	A	B OR C	DIFF.	1ST	2ND
Sc.	139.7	161.8	22.2	85.2	94.2	9.0	6	11
Ru.	224.2	216.3	-7.9	113.7	105.7	-8.0	13	6
Br.	183.1	198.3	15.1	92.0	93.6	1.6	7	7
Ca.	173.4	178.4	4.9	99.3	102.1	2.8	7	11
Ti.	179.3	187.5	8.1	107.3	112.7	5.4	6	13
Li.	164.6	177.8	13.2	99.0	106.3	7.3	5	21
La.	163.5	179.8	16.2	90.5	109.0	18.5	2	8
Ma.	160.4	158.3	-2.1	88.4	89.7	1.3	5	6
Average	173.5	182.3	8.7	96.9	101.7	4.7	6.4	10.4
s.d.			±10.1			±7.6		

of intermediate or unrestricted sodium excretion. Study of these twenty-one pairs (Table II) indicates that two consecutive periods on a like regimen of sodium intake were associated with an average recorded difference in blood pressure averaging 0.6 mm. of mercury diastolic and 1.1 mm. of mercury systolic. Considerable variation in the differences observed between like periods in each subject was noted here and also was present in the eight subjects comprising the experimental group.

TABLE II. AVERAGES OF BLOOD PRESSURES TAKEN DURING LIKE PERIODS OF SODIUM EXCRETION

SUBJECT	SYSTOLIC (MM. HG.)			DIASTOLIC (MM. HG.)			NUMBER OF ENTRIES	
	1ST	2ND	DIFF.	1ST	2ND	DIFF.	1ST	2ND
Sc.	156.8	164.7	7.9	92.8	95.0	2.3	4	7
Ru.	219.3	230.0	10.7	114.4	112.8	- 1.6	7	6
Br.	177.2	198.3	21.1	87.3	93.6	6.2	6	7
Ca.	178.6	178.0	- 0.6	101.9	102.5	0.6	7	4
Ti.	198.3	184.2	-14.1	120.0	110.5	- 9.5	3	10
Li.	176.9	182.0	5.1	107.4	106.7	- 0.7	7	7
La.	183.0	174.3	- 8.7	113.0	102.3	-10.7	5	3
Ma.	156.5	162.0	5.5	87.0	95.0	8.0	4	2
Sw.	193.1	194.8	1.7	110.6	113.8	3.3	7	6
Mc.	209.4	213.5	4.1	110.0	110.6	0.6	5	8
Ll.	223.3	228.2	4.9	128.1	128.2	0.0	8	6
Gap.	151.8	147.7	- 4.1	79.2	78.8	- 0.4	5	6
Ho.	173.3	172.2	- 1.1	99.2	96.2	- 3.0	6	6
Ko.	142.0	139.3	- 2.7	96.8	100.3	3.5	5	6
Ce.	205.3	208.0	2.7	112.8	106.6	- 6.2	6	5
Pe.	206.5	207.4	0.9	112.3	111.3	- 1.0	6	7
St.	182.3	175.8	- 6.5	114.0	114.4	0.4	4	5
Hi.	241.0	232.6	- 8.4	129.4	126.0	- 3.4	7	10
Co.	124.5	119.6	- 4.9	77.7	77.6	- 0.1	6	5
Ka.	165.8	161.3	- 4.5	90.6	87.8	- 2.8	5	6
Gab.	183.3	197.0	13.7	90.3	92.3	2.0	6	7
Average	183.3	184.3	1.1	103.6	103.0	- 0.6	5.7	6.1
s.d.			±8.2			± 4.5		

To determine the likelihood that the differences in the eight subjects of the experimental group might be due to chance alone, a "t" test of statistical reliability was applied<sup>c</sup> using the formula:

$$t_{21} = \frac{\sum dp}{\sqrt{\sum p \sum r^2/a}},$$

<sup>c</sup>We are indebted to Dr. L. J. Savage of the Department of Radiobiology and Biophysics, for direction, advice, and assistance with this analysis, and also for serving as a well-tempered foil to our efforts.

where  $d$  = experimentally observed difference in blood pressure between periods of unlike sodium excretion,

$p$  = weighting factor for  $d = \frac{n \times n'}{n + n'}$  in which  $n$  = the number of entries during the lactose period and  $n'$  = the number of entries during the sodium period,

$r^2$  = the similarly weighted square of the difference between pairs of like periods, and

$a$  = the number of pairs of like periods or 21.

This formula relates the experimental differences in the eight subjects to the variance of the blood pressure of the entire group of twenty-one subjects. The  $t$  values obtained for 21 degrees of freedom were 2.93 for diastolic pressure and 3.17 for systolic pressure. On this basis, the probability that a difference of the degree experimentally observed would occur by random variation alone is less than one in one hundred for both systolic and diastolic values.<sup>19</sup>

A tendency to lose weight was noted, probably because of fluid loss and the difficulty of maintaining caloric intake. Every effort was made to keep the weight loss minimal. Some subjects even gained in weight and these were not exclusively the ones who were unable to restrict their sodium intake. The average weight loss was greater (2.0 Kg.) in the twelve subjects whose sodium excretion was above 0.5 Gm. than the weight loss (0.1 Kg.) in the eight subjects who were able to rigidly restrict sodium. (Tables III and IV.)

Generally the patients who could adhere to the diet claimed the most subjective benefit; several could hardly be kept from continuing the regimen. One of these died abruptly three days after agreeing to liberalize her salt intake. The cause of death was presumed to be a cerebrovascular accident.

No evidence of undue salt deprivation was encountered. The study was not carried out during the summer months. It was the observers' impression that respiratory infections seem to be followed by undue degrees of asthenia. One patient developed pneumonia together with a condition resembling salt depletion. Her participation in the experiment was terminated and inquiry into the schedule revealed that she had been receiving supplemental salt. No abnormalities were noted in serum electrolytes in this or any other case.

Certain collateral information was obtained by analyzing the Clinic records of our subjects prior to and following the controlled experiment. Blood pressures taken by the same or by other observers and without uniform technique have been averaged for over three periods: (1) before 1943, (2) 1943 through 1945, and (3) 1946 and 1947 until the subject was selected for study. In certain instances the experiment was preceded also by a short period on diet alone and in some instances there was a further period of observation on unrestricted sodium intake at the conclusion of the study. The technique followed in these last two periods was essentially comparable to that used during the study except for the omission of the disguised sodium chloride or lactose.

TABLE III. COMPARISON OF PREVIOUS BLOOD PRESSURES WITH VALUES OBTAINED DURING CONTROLLED ADMINISTRATION OF MINIMAL SODIUM DIET GROUP I

PA- TIENT	AGE	SEX	WEIGHT CHANGE DURING EXPERIMENT (KG.)	BLOOD PRESSURE AVERAGES (MM. HG)							AVERAGE URINARY SODIUM (GM./24 HR.)						
				PREVIOUS RECORD			DIET ALONE*	EXPERIMENT PERIODS			OFF DIET†	A	B	C	OFF DIET†		
				BEFORE 1943	1943- 1945	1946- 1947		A	B	C							
Sc.	50	M	72.9 to 73.3	-	175 95	159 91	149 84	140 85	-	162 94	.73	.41	-	1.57	2.22		
Ru.	49	F	53 to 54.6	265 135	294 143	231 143	233 126	224 114	-	216 106	.16	.17	-	1.25	-		
Br.	56	F	53.6 to 53.4	-	-	214 100	198 96	183 92	177 87	198 93	.37	.38	.88	1.62	1.38		
Ca.	67	M	68.6 to 66.6	158 94	175 110	192 113	153 90	173 99	178 102	-	.10	.12	.91	-	1.06		
Ti. (Glomerulonephritis)	29	M	54.4 to 55.2	127 84	-	202 135	188 122	179 107	-	188 113	.16	.21	-	1.68	2.94		
Li. (Pyelonephritis)	48	M	70.4 to 72.5	-	177 111	191 112	175 114	165 99	-	178 106	1.13	.26	-	2.23	-		
La.	63	F	66.8 to 66.2	-	223 134	200 115	160 94	164 91	174 102	183 113	.36	.25	.89	1.33	-		
Ma.	57	F	59.3 to 56.6	-	185 97	205 110	175 100	160 88	-	158 90	.14	.27	-	1.61	-		
Average change -0.1				Average change from B. P. of 1946-1947			Average										
				-44 -20			2 1			-29 -15			.39 -12			.26 -5	
													.89 1.61			2.05	

\*1 to 4 weeks.  
†1 to 7 weeks.

TABLE IV. COMPARISON OF PREVIOUS BLOOD PRESSURE WITH VALUES OBTAINED DURING CONTROLLED ADMINISTRATION OF MINIMAL SODIUM DIET GROUP II

GROUP III

P.A. TUNT	AGE	SEX	WEIGHT CHANGE DURING EXPERIMENT (KG.)	BLOOD PRESSURE AVERAGES (MM. HG)							AVERAGE URINARY SODIUM (GM./24 HR.)						
				PREVIOUS RECORD				DIET ALONE*	EXPERIMENT PERIODS			OFF DIET†	DIET ALONE*	A	B	C	OFF DIET†
				BEFORE 1943	1943	1945	1946- 1947		A	B	C						
Sw.	54	F	70.9-73.6	105	186	185	-	-	-	193	-	-	-	-	-	1.70	-
				117	121	111	-	-	-	112	-	-	-	-	-	-	-
Mc.	56	M	70.5-72.8	-	189	209	200	200	209	212	223	1.05	-	.73	1.29	6.20	-
				-	99	106	100	100	106	110	116	-	-	-	-	-	-
Li. (Sympathectomized)	44	F	63.6-58.0	-	229	230	-	-	213	225	226	-	-	.85	1.76	3.41	-
				-	131	129	-	-	126	128	128	-	-	-	-	-	-
Ga.	39	M	95-85.5	-	-	183	151	151	162	150	144	.14	-	.39	2.30	4.96	-
				-	-	97	80	80	85	79	73	-	-	-	-	-	-
Ho.	54	F	64.9-63.0	195	201	212	172	172	174	173	197	.63	-	1.04	1.87	4.16	-
				108	110	117	100	100	101	98	110	-	-	-	-	-	-
Ko.	44	M	81.4-75.2	-	-	185	167	167	141	153	-	-	-	.80	1.50	-	-
				-	-	122	118	118	99	107	-	-	-	-	-	-	-
Ce.	70	F	74.3-76.7	213	220	226	173	173	208	205	-	.25	-	1.0	1.45	-	-
				117	123	123	100	100	107	113	-	-	-	-	-	-	-
Pe. (Sympathectomized)	53	F	89-86.4	210	227	232	199	199	-	208	210	1.09	-	-	2.44	4.43	-
				120	124	130	108	108	-	111	109	-	-	-	-	-	-
St.	51	F	77.9-71.2	-	-	187	162	162	180	180	190	.34	-	-	1.81	3.06	-
				-	-	121	108	108	116	121	121	-	-	-	-	-	-
Hi.	53	F	54.0-52.0	-	-	270	-	-	-	234	232	-	-	-	1.51	-	-
				-	-	142	-	-	-	127	119	-	-	-	-	-	-
Co.		M	72.1-73	150	129	133	119	119	-	124	123	.81	-	-	1.63	.99	-
				95	87	101	76	76	-	79	77	-	-	-	-	-	-
Ka.	55	F	61.5-56.9	-	-	174	166	166	-	164	153	1.43	-	-	1.74	1.23	-
				-	-	99	99	99	-	89	82	-	-	-	-	-	-
Average change from B. P. of 1946-1947				-14	-9	-27	-	-	-23	-19	-17	.72	-	.84	1.75	3.56	-
Average change				-5	-3	-14	-	-	-12	-11	-12	-	-	-	-	-	-
Group III				-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ga.	50	F	-	-	-	243	194	191	-	-	-	.213	.10	.05	-	-	-
				-	-	108	98	91	-	-	-	-	-	-	-	-	-

\*1 to 4 weeks.  
†1 to 8 weeks.

The average results of this evaluation are recorded in Tables III and IV. For comparison, the experimental data are entered according to the level of urinary sodium as A, B, or C. The cases are arranged into three groups. The first group (Table III) includes the eight patients who completed at least an A and a B or C period. The second group (Table IV) includes twelve patients with no A period, and the third group consists of a single patient with A periods alone. The change in blood pressure in the first two groups is averaged, using the value for 1946 and 1947 as a base. The averaged sodium excretion also is shown for the experimental periods. The experiment in the third group was terminated by intercurrent illness. The comparison reveals an apparent fall in blood pressure in all groups during the periods of study. This fall is of a magnitude much greater than that experienced within the controlled portion of the experiment. For the study periods of lowest averaged blood pressure, in the eight experimental subjects used for evaluation of sodium restriction the average fall in systolic pressure was 30 mm. of mercury and in diastolic pressure 20 mm. of mercury. A similar comparison for the group of patients who did not follow a period of strict sodium limitation as defined shows an average maximum fall of 28 mm. of mercury systolic, and 16 mm. diastolic. There is a general tendency for the pressure to vary with the salt excretion. (It must be recalled that periods A, B, and C were not necessarily consecutive in any patient.) However, the sodium excretion after termination of the experiment was comparable to that of an unrestricted diet and, nevertheless, blood pressure average remained below the 1946-1947 level for both groups.

#### DISCUSSION

The average of the differences in blood pressure which can be ascribed to sodium restriction alone under the conditions of the study is shown to be quite small, 9 mm. systolic and 5 mm. diastolic. It should not be inferred from this that a regime of sodium restriction is ineffective or unwarranted therapy in uncomplicated hypertensive vascular or cardiovascular disease. Two important considerations should be emphasized in this regard.

(1) While the average response in the group of patients studied is small, certain individuals showed a greater response than others. Patient La., on the controlled portion of the study, evidenced a fall in diastolic pressure of 19 mm. of mercury. A fall of between 12 and 22 mm. of mercury in systolic pressure was evidenced in four cases. It is probable that there are individual differences in responsiveness among patients. Perhaps, also, the degree of response to sodium restriction is modified by other factors not herein controlled. It is to be emphasized that there may be different varieties of the disorder which is called "essential" hypertension in our ignorance of its homogeneity or etiology. When a large number of patients has been examined and the responsiveness of these patients to variation in sodium intake alone has been analyzed it may be possible to group the type of patient who responds into a separate category. The size of the present study does not permit deductions of this nature. Nor can we identify our responsive patients as being of the "pseudo-Cushing's" type reported by Schroeder.<sup>12</sup>

(2) It will be noted by comparing the collaterally obtained information with that obtained during the experimental part of the study, that all patients, even those who responded very little to restriction of sodium alone, had a reduction in blood pressure over the period encompassed by the study. This maximum change in pressure is the figure that would be obtained from an entirely "clinical" evaluation of these patients and represents the overall effect of all procedures used, plus the spontaneous variations in blood

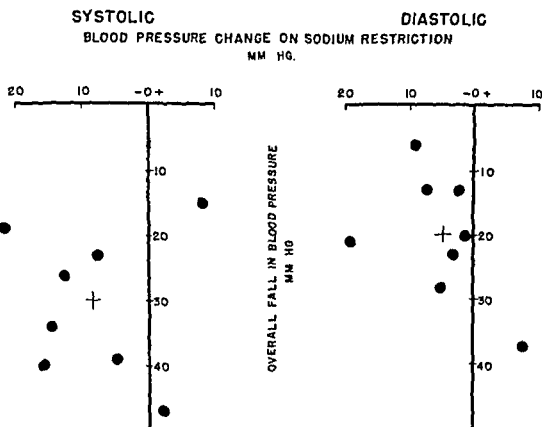


Fig. 1.—For the eight subjects in whom the effect of sodium restriction alone can be determined, the blood pressure change ascribed to this restriction is compared with the maximum fall in pressure from the clinical records. Each dot represents one subject; the cross represents the average of all subjects. (All illustrations courtesy of the Veterans Administration, Washington, D. C.)

pressure, differences between observers, etc. If no analysis had been made of the change in blood pressure associated with sodium restriction alone, this entire effect could have been ascribed mistakenly to the therapeutic value of sodium restriction. In Fig. 1, the fall in blood pressure ascribed to sodium restriction alone is compared with the maximum clinical fall in blood pressure based on the records. It will be noted that there is no correlation between the two sets of observations. Several individual records are presented in Figs. 2 to 8. One patient whose blood pressure rose during the period of sodium restriction as compared with a period of liberal administration of sodium nevertheless had the greatest fall in blood pressure of any of the group on an over-all basis (Fig. 2). Moreover, this patient claimed great symptomatic improvement on this regimen. Her blood pressure was lowest during the six week period in which the urinary sodium averaged 1.25 Gm. per day (equivalent to 3.2 Gm. of sodium chloride). The absence of correlation between the fall ascribable to sodium restriction alone and the general reduction in blood pressure that occurred during the course of the study,

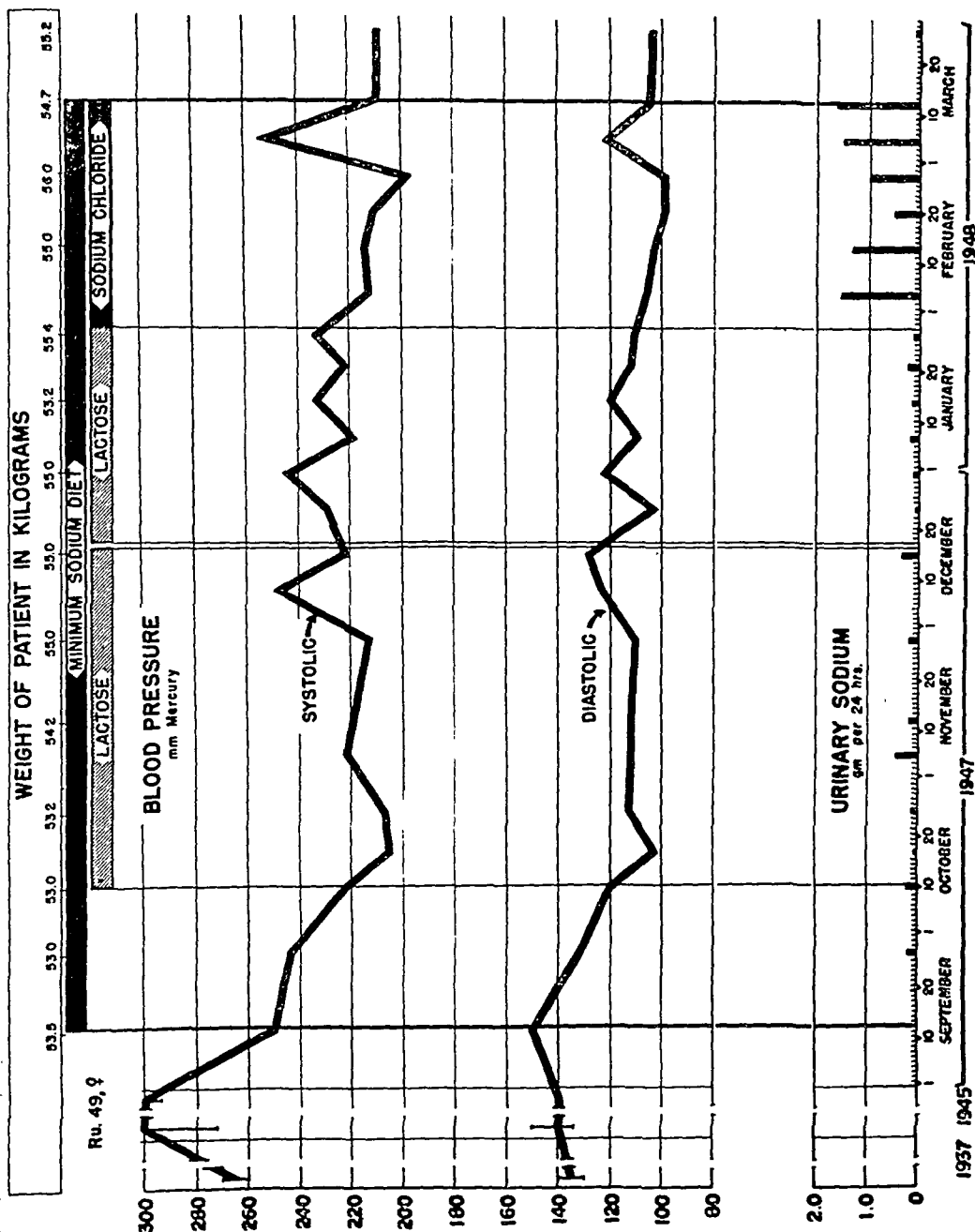


Fig. 2.—Patient Ru. This woman had long-standing, marked hypertension. On numerous occasions her systolic pressure was too high to be recorded. Although her blood pressure fell during sodium restriction, it was lowest during the last period when salt was substituted for the lactose. Her symptomatic relief was so striking that she was unwilling to relinquish the diet. Death occurred in April, 1948.

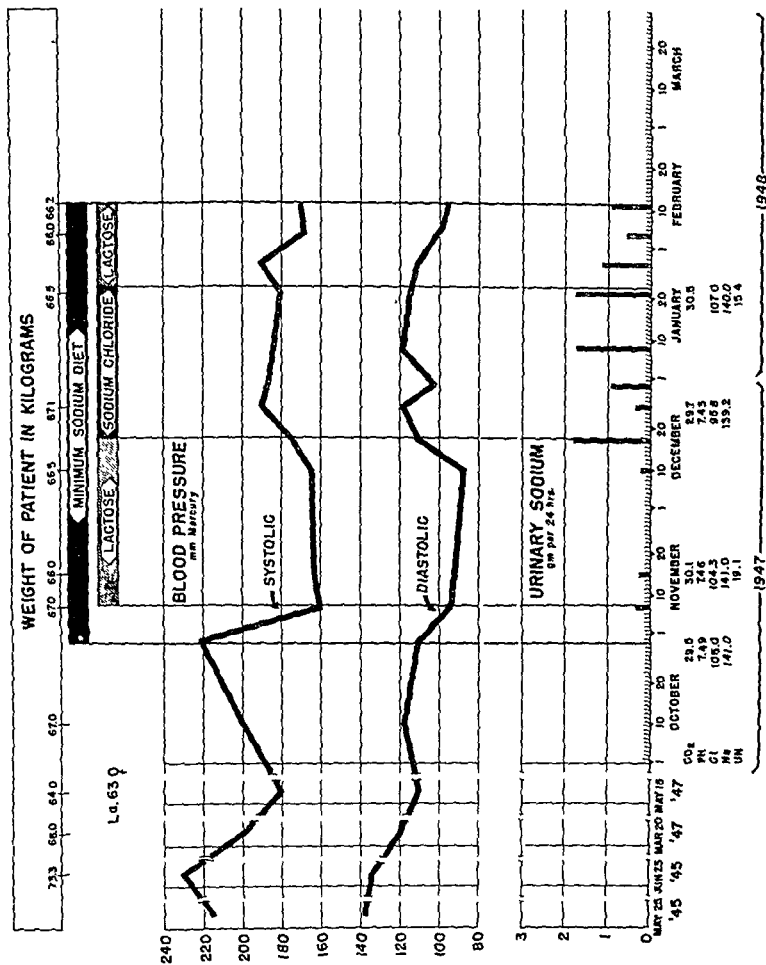


Fig. 3.—Patient La., with previously recorded diastolic pressures over 110, showed a fall to norm 1 levels during sodium restriction and a rise during supplementation with salt. Little weight change occurred.



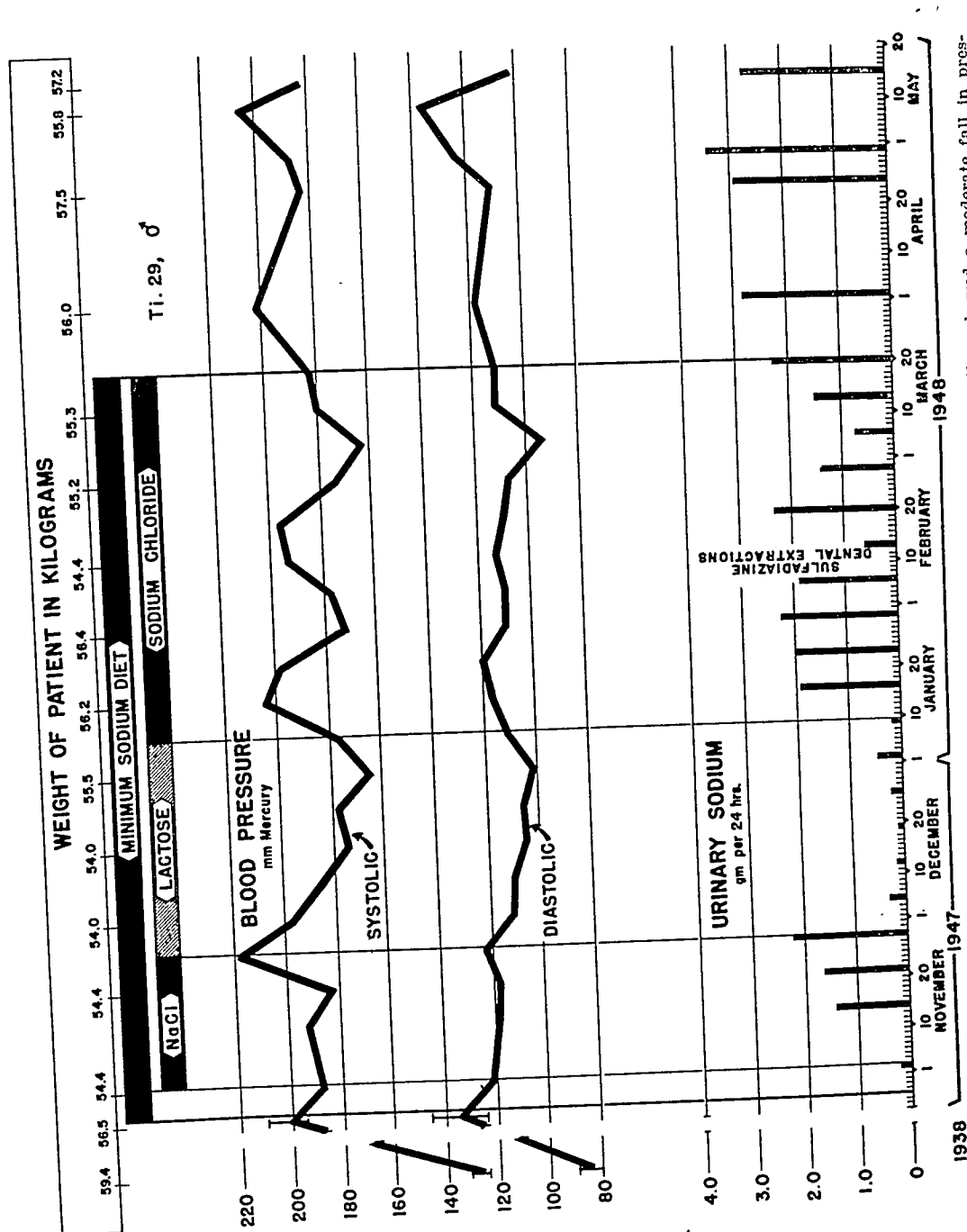


Fig. 4.—Patient Ti., a young man with glomerulonephritis of less than nine years' duration, showed a moderate fall in pressure during sodium restriction but maintained this level of blood pressure despite supplemental salt.

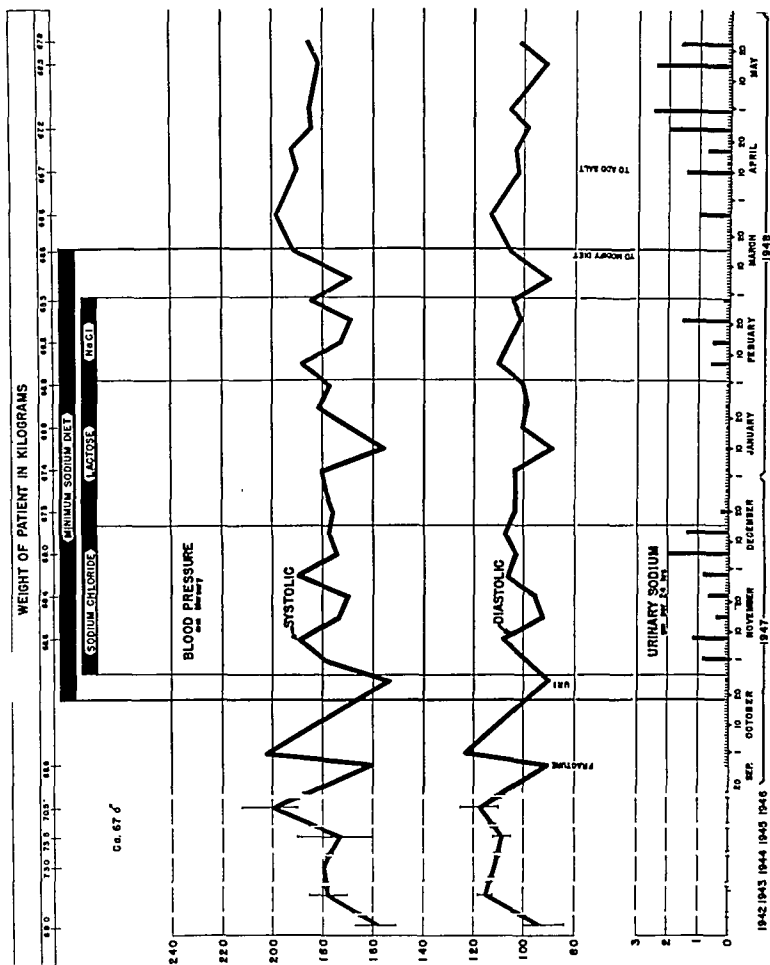


Fig. 5.—Patient Ca., an elderly man who adhered to his diet strictly, showed no effect of salt restriction upon his blood pressure, although he claimed great symptomatic benefit.



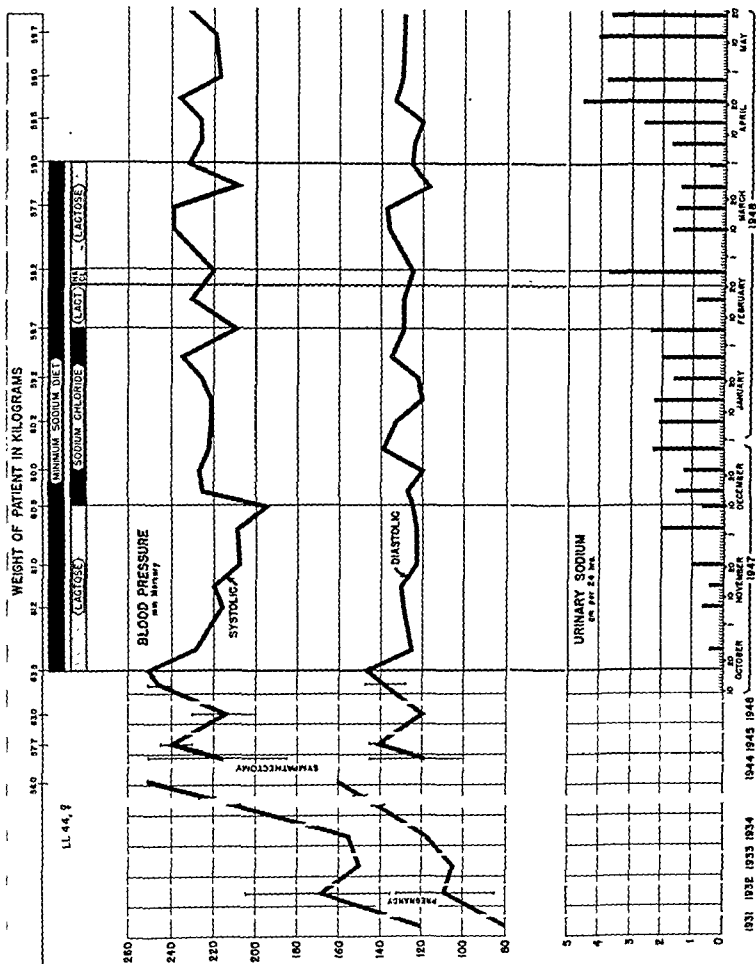


Fig. 7.—Patient L.L. had been observed for sixteen years. The onset of her hypertension dated to pregnancy. Subsequent acceleration was followed by a Smithwick sympathectomy in 1944. Her inability to remain on the diet invalidates the attempted evaluation of the effect of sodium restriction upon her blood pressure.

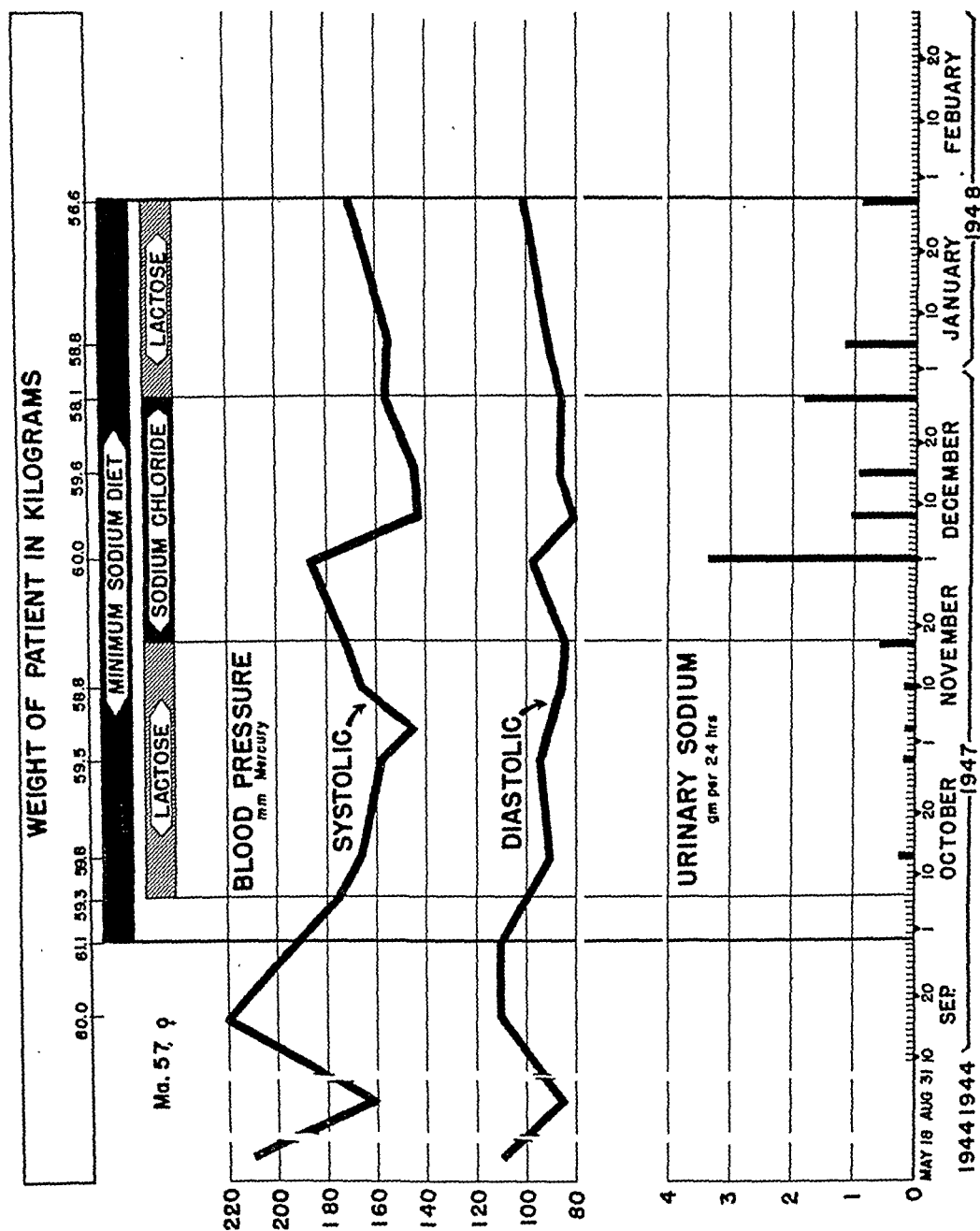


Fig. 8.—Patient Ma. showed a generally somewhat lower pressure during the study period than before. Rigid sodium restriction for six weeks was without added effect however.

and the small number of cases of the experimental group, make it rather pointless to present the individual cases in a search for the factors which make some patients susceptible to sodium restriction and others not. If the effect of this regimen is considered as the resultant of the two components, i.e., the effect of sodium restriction per se and the effect of all other factors, probably variation exists between individuals in each of these effects. It would be important to know how to select those patients who might have a good response to sodium restriction. It would appear of even greater importance to have criteria for selection of patients who respond to the factors other than sodium restriction. Both necessitate extension of our studies.

The foregoing comments deal only with the response of the blood pressure under the described regimen. A further factor of therapeutic importance arises when it is considered that the symptomatic effect of this regimen did not always parallel the change in blood pressure, nor the variation in sodium intake. There were patients who felt better although the blood pressures did not fall, and there were patients who felt better during a period when the sodium intake was higher than it had been before, regardless of any change in blood pressure. Our brief studies do not permit analysis of these relationships nor do they control spontaneous variations in the level of the patient's symptoms.

We have been quite arbitrary in selecting the level of sodium excretion by which our patients have been grouped. Previous workers have maintained that a urinary sodium or chloride excretion equivalent to more than 0.5 Gm. of sodium per day should not be considered an adequate degree of sodium restriction for the treatment of hypertension. It is quite possible that the blood pressure in some patients may fall with much more moderate sodium restriction than that which we have attempted. The average blood pressures for periods at different levels of sodium excretion reveal to some extent the individual and average response of patients when the degree of sodium restriction is varied. A more important objection to the conclusions of the study lies in the fact that the period of observation was arbitrarily limited to six weeks. In several instances it appeared that a change in blood pressure was becoming manifest toward the end of the six-week period. This is in accord with the results of others who have pointed out that a delay in response may be noted. This is said to occur particularly during desalting. Such a phase displacement, commented on previously, will minimize any apparent effect of sodium restriction alone in this study. We do not feel that our data permit division of the experimental periods each into two periods of three weeks, although this would be a way of approaching this valid criticism.

The study was designed to evaluate only one point, and that was whether sodium restriction *alone* was capable of significantly reducing the blood pressure of hypertensive patients. Here "significant" does not mean the significance to the patient or any therapeutic significance but simply whether sodium restriction had a *statistically significant* effect upon the blood pressure. This must be established before one can view in its proper light the result of the

complex alterations which ensue when a patient comes to the doctor's office or to the clinic and is advised to go on a rigidly restricted diet. It is important for the doctor to know whether the diet he advises is effective because of its sodium restriction, because of its specific composition, because of its psychological implication, or whether the patient appears to benefit because some active therapeutic effort is being made in his behalf or simply because the doctor is taking an interest in him. From the point of view of some patients (and physicians), the mechanism may not seem to be of importance, it often sufficing that the patient feels better and that the response satisfies his physician in whom he places his trust to keep him well. For the physician, this is self-deception.

There is great need for a wider realization of the profound physiologic, psychologic, psychiatric,<sup>20</sup> social, and economic alterations which may result from a diet such as this, apart from the biochemical and physiologic effects of sodium chloride depletion.

#### SUMMARY

An attempt was made to provide a maintenance diet with a sodium content restricted to less than 300 mg. per twenty-four hours. This diet was offered to twenty-four ambulatory hypertensive patients on an experimental basis. In addition the subjects received either 4 Gm. of sodium chloride or lactose in identical form over three periods of approximately six weeks each according to planned schedules. Neither the subjects nor the investigators were aware of the schedule being used and the subjects were not aware of the nature of or alteration in the medication. The criterion adopted for rigid restriction was the average excretion of less than 500 mg. of urinary sodium in a weekly twenty-four hour sample. Blood pressures were recorded by a standardized technique approximately once each week. Eight subjects presented data for a period of restriction which could be compared with a period of greater sodium excretion. In these subjects the average diastolic blood pressure was 4.7 mm. of mercury lower and the average systolic pressure 8.7 mm. of mercury lower during the restricted period than during the period of sodium supplementation. Twenty-one subjects yielded data for at least two periods of a like order of sodium excretion from which the variances of the blood pressure for the group were determined. On this basis the probability that a difference as large as that experimentally observed would occur by random variation is less than one in one hundred for both the diastolic and the systolic values.

#### CONCLUSIONS

1. A diet rigidly restricted in sodium is difficult to administer successfully to ambulatory hypertensive subjects.
2. A difference of less than 5 mm. of mercury in the average diastolic blood pressure was observed, which could be ascribed to the effect of the sodium chloride restriction alone.
3. This difference cannot reasonably be assigned to random variation.

4. Reduction in blood pressure observed when hypertensive patients are placed upon a diet which is restricted in sodium may be only partly due to sodium restriction. Some of the other factors which may be involved are pointed out but not analyzed in this study.

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# INFLUENCE OF VARIOUS DISEASE STATES UPON THE FEBRILE RESPONSE TO INTRAVENOUS INJECTION OF TYPHOID BACTERIAL PYROGEN

WITH PARTICULAR REFERENCE TO MALARIA AND CIRRHOSIS OF THE LIVER

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CLINICAL experience during fever therapy has shown that patients given a series of injections of a bacterial pyrogen, such as that present in typhoid vaccine, develop a remarkable tolerance to the pyrogen, so that increasing doses must be given in order to produce comparable elevations in body temperature. This type of immune reaction or tolerance is of short duration and is apparently not dependent on the presence of specific antibodies.<sup>1, 2</sup> Experiments in rabbits indicate that it is related to an increased ability of the reticulo-endothelial system to remove the pyrogen from the circulation.<sup>3</sup> The question arises whether a similar tolerance develops in human beings during the course of certain febrile diseases. If such alterations do occur, patients convalescent from these diseases would be expected to show very little febrile reaction to intravenous injection of a bacterial pyrogen. One observation pertinent to this problem was reported by Howard<sup>4</sup> who, in studying the effect of fever on protein metabolism, found that he was unable to elicit an appreciable febrile response to typhoid vaccine in a patient who had recently recovered from malaria. Morgan<sup>5</sup> reported that patients convalescent from typhoid and paratyphoid fever showed no significant febrile reactions to intravenous injection of purified extracts of *Eberthella typhosa* and *Shigella dysenteriae*. This material, however, produced marked febrile reactions in normal individuals. The present report summarizes observations on the febrile response of patients with various diseases to intravenous injection of a small dose of typhoid vaccine.

## METHOD

The vaccine employed was a suspension of heat-killed *E. typhosa* containing approximately one billion organisms per milliliter. The standard dose was .05 ml. injected intravenously. Previous experience with this vaccine had shown that this dose produced little symptomatic discomfort other than a sensation of chilliness followed by a rise in temperature of 1.5° F. to 3° F., with temperature returning to normal in four to six hours. Temperature measurements were made with a constant-recording rectal thermometer. Readings were begun one hour previous to the injection and continued at thirty-minute intervals for eight hours afterward. The febrile response was plotted on graph paper and the temperature previous to injection was taken as the base line. The area between the base line and the course of the febrile response was measured with a planimeter. The size of this area, expressed in vernier units of the planimeter, was called the "fever index."

The subjects studied can be divided into five main groups.

1. A "normal" group of sixteen afebrile patients receiving penicillin treatment for asymptomatic neurosyphilis.

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2. Twenty-two patients convalescent from a variety of acute infectious diseases, including seven with pneumonia, four with gonorrheal arthritis, three with typhus fever, three with tularemia, two with typhoid fever, two with meningitis, and one with rheumatic fever. The vaccine was administered to these patients two or three days after they had recovered from their illnesses and had become afebrile.

3. Twenty patients who had just completed a course of malarial fever therapy for neurosyphilis. The test was carried out on the second or third day following the last malarial paroxysm.

4. Fourteen patients with cirrhosis of the liver.

5. Thirteen patients with jaundice, ten of whom had viral hepatitis, and three of whom were thought to have biliary obstruction. All of the patients with liver disease were tested at a time when their temperatures had been normal for two or three days.

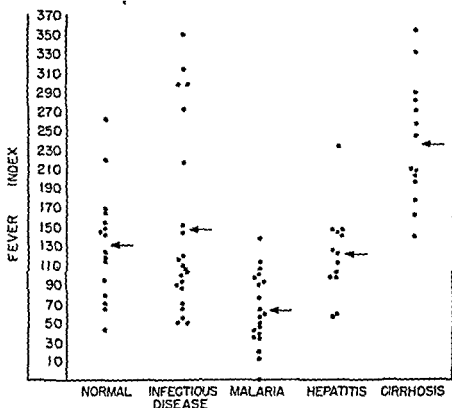


Fig. 1.—The febrile response to typhoid vaccine in various disease states. The arrow indicates the mean response in each group of patients.

## RESULTS

The febrile responses following injection of typhoid vaccine in the different groups are shown in Fig. 1. It will be observed that in the "normal" subjects the range of the fever index varied from 43 to 262, the mean being 132. This quantitative expression of both the height and the duration of fever indicates a rise of 1.5° F. to 3° F., the temperature returning to normal usually within six hours.

In patients recently convalescent from acute infections, the febrile responses were comparable to those of the "normal" subjects, the mean value of the fever index in this group being 148. The four highest values were in two patients with typhoid fever and two with tularemia. The two patients with typhoid fever, the only ones in this study with this condition, exhibited

an increased sensitivity to pyrogen, a finding at variance with that of Morgan<sup>5</sup> who reported a decreased febrile response in a larger number of patients convalescing from this illness. One other patient with tularemia showed a febrile response similar to that appearing in the normal subjects.

The twenty patients who had recently recovered from malaria reacted to the typhoid vaccine with less fever than the preceding groups, their mean fever index being only 63. This finding is similar to that noted by Howard in one case. Comparable observations were made by Habetin,<sup>6</sup> who found that the injection of pyrogenic nuclein, made from yeast, failed to produce any febrile response in a few patients with malaria.

Patients with cirrhosis of the liver showed greater than normal responses, the fever indices ranging from 140 to 352, the mean value being 236.

In the group of thirteen patients with jaundice, twelve showed responses within the "normal" range, whereas one with infectious hepatitis had an exaggerated febrile response. This person had been ill for approximately four months, and liver function tests showed considerable evidence of hepatic insufficiency at the time the test was performed.

#### DISCUSSION

The increased sensitivity to pyrogen observed in patients with chronic liver disease may possibly be caused by impaired reticuloendothelial function in the liver and spleen, or may be related to some metabolic disturbance. Patients with cirrhosis have been observed to develop fever following the intravenous administration of salt-poor albumin.<sup>7</sup> There is also a definite impression that certain preparations of albumin produced febrile reactions in persons with cirrhosis, but not in patients with hypoalbuminemia due to other causes.<sup>8</sup> The patients in the present study with acute hepatitis or obstructive jaundice, however, did not exhibit abnormal responses to typhoid vaccine. Experiments with rabbits in this laboratory have also shown that *acute* liver damage, produced by chloroform, did not lead to abnormal febrile response to typhoid pyrogen.

The diminished response to the pyrogen in patients with malaria may possibly be associated with the great proliferation in reticuloendothelial elements known to occur in this infection. The stimulation of the reticuloendothelial system produced by malarial fever and typhoid vaccine fever therapy has been offered as an explanation for their curative effect in neurosyphilis.

The patients in this study with febrile diseases other than malaria developed little if any tolerance to bacterial pyrogen. Morgan,<sup>5</sup> however, has observed that patients convalescent from typhoid and paratyphoid fevers showed tolerance to injection of purified extracts of typhoid and dysentery bacilli, whereas the reaction observed in five patients recovering from other febrile diseases was comparable to that of normal subjects. Bennett found that rabbits surviving pneumococcal and colon bacillus infection did not show a diminution in febrile response to typhoid or colon bacillus pyrogens.<sup>9</sup>

## SUMMARY

The febrile response to typhoid bacterial pyrogen was observed in a group of eighty-five patients with various diseases. The mean response elicited by patients with a variety of infections, such as pneumonia, hepatitis, typhus fever, and tularemia, was the same as that observed in normal individuals. Patients with malarial fever, however, appeared to be comparatively resistant to the pyrogen. Patients with cirrhosis of the liver exhibited an exaggerated response.

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# THE IN VIVO ACTION OF AUREOMYCIN ON PLEUROPNEUMONIA-LIKE ORGANISMS ASSOCIATED WITH VARIOUS RHEUMATIC DISEASES

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THE efficacy of gold compounds in animals experimentally infected with animal strains of pleuropneumonia-like organisms has been demonstrated by various observers.<sup>1-6</sup> A strain of *Streptobacillus moniliformis* recovered from the joint fluid of a patient produced arthritis in mice with great regularity. Arthritis could be prevented by the simultaneous administration of gold and the infecting agent, but arthritis once established was unaffected by the use of gold. The transformation of this strain of *S. moniliformis* into the pleuropneumonia-like form was readily demonstrated.<sup>7</sup>

A patient with nonspecific urethritis and prostatitis from whom pleuropneumonia-like organisms were recovered has been treated with Myochrysin without improvement.<sup>8</sup> Sulfonamides and penicillin have been found ineffective both in vitro and in vivo in animal and human infections with these organisms.<sup>7, 8, 9</sup> Streptomycin has been observed to have an effect on L organisms and has produced a rapid disappearance of symptoms in patients with an associated infection due to these organisms.<sup>8, 10</sup> However, pleuropneumonia-like organisms have been observed to persist in a case of nonspecific urethritis following streptomycin therapy.<sup>8</sup> It has been pointed out repeatedly that L organisms may be of significance in human joint diseases but a definite correlation has not yet been established.<sup>8, 11, 12</sup>

## OBSERVATIONS

In the present study, patients with rheumatoid arthritis, from whom cultures of prostatic secretion yielded pleuropneumonia-like organisms, were observed for one year or more. An effort was made to determine whether or not under prolonged gold therapy for the rheumatoid arthritis the organisms could be eliminated in vivo in human beings. In all patients of this group, L organisms could be recovered with great regularity in spite of continued gold therapy and satisfactory clinical remissions.

In one of these patients, each of fourteen successive prostatic cultures obtained over a period of eighteen months was positive for L organisms. After thirteen months of gold therapy (10 mg. the first week, 25 mg. the second week, 50 mg. weekly for nineteen weeks; 50 mg. monthly for eight months; total dosage, 1,385 mg.), myochrysin was discontinued because of toxicity. Three

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months after cessation of therapy the rheumatoid arthritis relapsed and aureomycin\* therapy was instituted. There was a complete disappearance of pleuropneumonia-like organisms on six successive culture attempts during and following aureomycin therapy.

Disappearance of L organisms from the genital tract under aureomycin therapy has been demonstrated in a total of nine patients (three men, six women; two cases of erythema nodosum, one of rheumatic fever, five of rheumatoid arthritis, one of nonspecific urethritis). In two of the female patients L organisms reappeared after discontinuation of the drug, indicating either insufficient dosage or bacteriostatic rather than bactericidal activity of aureomycin. One of these patients was re-treated with aureomycin and again prompt elimination of the organisms was achieved.

Four patients with nonspecific urethritis have been treated with aureomycin. Two of these patients had joint and muscle complaints in addition to the urethritis. In only one patient cultures of prostatic secretion yielded abundant growth of pleuropneumonia-like organisms. There was prompt response to aureomycin (2.0 Gm. per day for four days), confirming the observation of Collins and co-workers.<sup>13</sup> Complete relief of symptoms occurred after forty-eight hours of therapy. Cultures taken thirty-six hours and one, ten, and eleven weeks after institution of aureomycin therapy were negative for pleuropneumonia-like organisms. In spite of negative cultures the patient had a recurrence of burning on urination but no return of urethral discharge. The burning sensation has persisted in the absence of further treatment. This observation, a recurrent clinical symptom and negative cultures in a patient in whom L organisms were demonstrated previously, supports the impression that absence of demonstrable L organisms does not rule out the possibility that disease was initiated by infection with them. The inability to demonstrate pleuropneumonia-like organisms may be due to the inadequacy of the present culture methods. This failure to culture organisms may also parallel the findings in *S. moniliformis* infections, in which in the later stages of the disease it may be impossible to recover the organism although clinical activity persists.<sup>7</sup> These speculations would explain the frequently noted lack of correlation between clinical and laboratory findings.

In three of the four patients with nonspecific urethritis, pleuropneumonia-like organisms could not be demonstrated in repeated cultures prior to aureomycin therapy. One patient was treated for an attack of three months' duration which had not improved under penicillin therapy. One gram of aureomycin was given daily for seven days. The patient was asymptomatic on the last day of treatment. Burning on urination recurred two weeks after cessation of therapy, and four weeks after discontinuation of aureomycin morning discharge was again present. A second course of aureomycin was given consisting of 1 Gm. daily for five days and 2.0 Gm. daily for three weeks. The discharge ceased after five days of therapy; burning diminished during treatment and cleared completely a few days after cessation of therapy. The patient has remained asymptomatic during a five-week follow-up period. A second patient

\*We are indebted to the Lederle Laboratories for part of the aureomycin used in this study.

TABLE I. EFFECT OF AUREOMYCIN THERAPY ON RHEUMATOID ARTHRITIS AND MARIE-STRÜMPPELL SPONDYLITIS

DIAGNOSIS	PATIENT	SEX	DEGREE OF INVOLVEMENT	DURATION OF ILLNESS	COURSE OF ILLNESS	AUREOMYCIN		AUREOMYCIN SERUM LEVEL ( $\mu\text{g./c.c.}$ )	THERAPEUTIC RESPONSE	L. ORGANISMS	
						GM./DAY	DURATION (WEEKS)			BEFORE THERAPY	AFTER THERAPY
Rheumatoid arthritis*	N. J.	F	Early Class II	2 yr.	Chronic, progressive	2.0	4	5.12	Complete remission	0	0
	C. W.	M	Early Class II	3 yr.	Chronic, intermittent	2.0	5	5.12	Major improvement	0	0
	F. W.†	M	Early Class III	4 yr.	Intermittent, acute exacerbation	2.0	7	1.28	Unimproved	0	0
	F. N.	M	Early Class III	6 mo.	Progressive	2.0	10	-	Major improvement	0	0
	L. T.	F	Early Class II	5 yr.	Chronic, acute exacerbation	2.0	8	-	Complete remission	-	-
	B. S.	F	Early Class III	12 yr.	Intermittent, acute exacerbation	1.0 2.0	2 12	-	Major improvement	+	0
	J. Y.	M	Moderate Class III	3 yr.	Intermittent, acute exacerbation	1.0 2.0 2.5	8 2 4	- - 0 0.32	Unimproved	+	0 0 0
	L. C.	F	Moderate Class II	12 yr.	Chronic, intermittent	2.0	8	0.64	Major improvement	+	0
	R. H.	F	Moderate Class IV	3 yr.	Chronic, intermittent	2.0	2	-	Minor improvement	+	0
	J. G.	M	Moderate Class III	6 yr.	Chronic, progressive	0.5 1.0	3 12	- 2.56	Major improvement	+	0
	M. F.	F	Moderate Class II	20 yr.	Chronic, progressive	1.0 1.5 2.0	7 4 7	- - -	Unimproved Unimproved Major improvement	0	0
	E. T.	M	Severe Class III	7 yr.	Chronic, progressive	1.0	16	2.0	Major improvement	0	0
	E. V.	F	Terminal Class III	11 yr.	Chronic	1.0	15	Joint fluid level 2.0 0.08	Minor improvement	0	0

Marie-Strumpell spondyli- tis	H. H.	M	Moderately severe	6 yr.	Chronic	4.0 2.0	1 5	10.24 5.12	Good	0
	O. T.	M	Moderately severe	4 yr.	Chronic, progressive	2.0	8	10.24 2.56	Good	0
	A. J.	M	Moderately severe	4 yr.	Chronic	2.0	6	5.12 5.12	Good	0
	L. A.	M	Moderately severe	6 yr.	Chronic, intermittent, slowly progressive	2.0	5	1.28 0.32	Unimproved	0
	J. H. B.†	M	Severe	2 yr.	Chronic, progressive	2.0	9	2.56 2.56 0.64	Good	0
	D. T.†	M	Severe	7 yr.	Chronic, progressive	2.0	8	0 1.28 0.32 0.16	Unimproved	0
	J. B.‡	M	Severe	20 yr.	Chronic, acute	2.0	5	-	Temporary improve- ment followed by exacerbation	0

Minus sign denotes that the determination was not made.

\*Method of classification of rheumatoid arthritis according to criteria adopted by American Rheumatism Association.<sup>10</sup>

†Atypical rheumatoid arthritis.

‡With peripheral joint involvement.



became asymptomatic following forty-eight hours of treatment (1.0 Gm. per day for seven days), but mild burning on urination developed one week after aureomycin had been discontinued. This patient has not been re-treated. The third patient presented a two-year history of urethritis. Burning and urethral discharge were present at the onset of the disease. After three months of the illness the discharge cleared spontaneously, but burning and frequency of urination persisted with the gradual development of arthralgia, myalgia, and fatigue. Aureomycin therapy (0.5 Gm. per day for four weeks, 1.0 Gm. per day for ten days) appeared ineffective. However, when the dose of aureomycin was increased to 2.0 Gm. per day striking improvement was noted. After the patient had been on this larger dosage for three weeks, he noted for the first time in two years absence of burning on urination and was completely free of myalgia, joint pain, and fatigue. Treatment was continued for a total of ten weeks. There was no recurrence of symptoms. There has been inadequate time for follow-up studies.

Twenty-five patients representing a variety of rheumatic diseases (rheumatoid arthritis, Marie-Strümpell spondylitis, rheumatic fever, erythema nodosum) have been treated with aureomycin (Tables I and II).<sup>14</sup> Cultures from the cervix and prostatic secretions yielded pleuropneumonia-like organisms in eight of these patients. Cultures were negative for L organisms in seventeen patients.

Subjective and objective improvement was observed in seventeen of these twenty-five patients. Two (Patients R. H. and E. V.) were symptomatically improved. One case of rheumatic fever (Patient J. T.) could not be evaluated because salicylates were given simultaneously. Two patients with Marie-Strümpell spondylitis (Patients D. T. and L. A. showed no demonstrable improvement after five and eight weeks of treatment. One individual with Marie-Strümpell spondylitis and peripheral joint involvement (Patient J. B.) experienced temporary marked symptomatic improvement followed by an acute

TABLE II. EFFECT OF AUREOMYCIN THERAPY ON RHEUMATIC FEVER AND ERYTHEMA NODOSUM

DIAGNOSIS	PATIENT	SEX	DEGREE OF INVOLVEMENT	DURATION OF ILLNESS	AUREOMYCIN		THERAPEUTIC RESPONSE	L ORGANISMS	
					GM./DAY	DURATION (DAYS)		BEFORE THERAPY	AFTER THERAPY
Rheumatic fever	J. T.	F	Chronic and acute, severe	14 yr.	2.0	17	?	+	0
	S. P.	F	Chronic and acute, severe	7 yr.	0.75	45	Good	-	
	P. W.	M	Acute, severe	7 yr.	2.0	10	Good	0	
Erythema nodosum with fever and joint involvement	C. M.	F	Acute, severe	2 mo.	2.0	5	Good	+	0
					1.0	11			
Erythema nodosum with fever	N. C.	F	Acute, severe	2 mo.	0.5 1.0	5 20	Good	+	0

Minus sign denotes that the determination was not made.

exacerbation of the disease. Two persons (Patients F. W. and J. Y.) with rheumatoid arthritis did not improve. It may be of significance (Table I) that the aureomycin blood serum levels were uniformly low in those patients in whom little or no clinical improvement was noted.

The relation between dosage of aureomycin, aureomycin blood serum levels, temporary or permanent elimination of L organisms, and clinical response will require further study. Disappearance of L organisms in the patients with positive cultures was more rapid than the clinical improvement noted in any single case with the dosage of aureomycin and the route of administration employed. In one case of erythema nodosum, as little as 0.25 Gm. of aureomycin daily was all that was necessary to eliminate pleuropneumonia-like organisms from the cervix. In this case, 1.0 Gm. per day of the antibiotic was required to produce a favorable clinical response. One patient in whom L organisms disappeared promptly failed to show clinical response. The patients with negative cultures for L organisms responded as well as those from whom the organisms could be recovered.

There was an initial exacerbation of the illness in a number of cases of rheumatoid arthritis and Marie-Strümpell spondylitis comparable with that frequently observed by us in rheumatoid arthritis treated with gold. This exacerbation of muscle and joint symptoms varied in intensity and duration, but uniformly disappeared under continued therapy. Two patients who had fever prior to therapy developed an initial, transient, additional rise in temperature on 2.0 Gm. of aureomycin daily. The three other febrile patients in the rheumatic fever-erythema nodosum group, in whom aureomycin did not exceed 1.0 Gm. per day, did not have this additional febrile response.

It was observed early in the course of this study that some patients who failed to improve on 1 Gm. of aureomycin daily responded favorably when the dosage was increased to 2.0 Gm. per day. Therefore, the 2 Gm. dose was selected whenever the patient could tolerate this amount. In most patients improvement was noted one to four weeks after initiation of therapy and continued in a manner comparable to that observed with successful gold therapy of rheumatoid arthritis. The high incidence of favorable response to aureomycin suggested an effect which was more than a coincidental finding. Aureomycin therapy may not be suitable in cases of rheumatic fever because of the initial exacerbation of symptoms noted. Smaller initial doses than those employed in the present study may eliminate this exacerbation.

Kuzell<sup>15</sup> has reported that the effect of aureomycin was similar to that of gold in the control of rat arthritis experimentally produced with the L<sub>4</sub> strain. Brown<sup>16</sup> has noted the effect of aureomycin in human beings with rheumatic diseases. It would appear that aureomycin and undoubtedly other antibiotics with a similar effect on L organisms may assume an essential role in the understanding of the relationship of pleuropneumonia-like organisms to rheumatic diseases. Preliminary studies indicate that the effect of chloromycetin may be comparable to that of aureomycin. In one female patient with severe, progressive rheumatoid arthritis, repeated cervical cultures were done before and

after administration of 1.0 Gm. of chloromycetin daily. Prompt elimination of L organisms was achieved. There was an exacerbation of symptoms followed by rapid major clinical improvement within three weeks.

#### SUMMARY

1. Oral administration of aureomycin has produced uniform disappearance of demonstrable pleuropneumonia-like organisms from the genitourinary tract of individuals with and without joint disease. L organisms were not eliminated by gold therapy.

2. Elimination of L organisms by aureomycin or by other antibiotics producing a similar effect may be of importance in the understanding of the possible relationship of pleuropneumonia-like organisms to articular diseases.

3. The effect of aureomycin therapy in twenty-five cases of rheumatic disease has been reported.

4. The ultimate value of aureomycin in the treatment of rheumatic diseases must await prolonged clinical trial and follow-up study.

We are indebted to Dr. W. D. Jarman for permitting the use of his case in this study (one case of nonspecific urethritis).

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# SONIC-VIBRATED LEPTOSPIRAE AS ANTIGENS IN THE COMPLEMENT FIXATION TEST FOR THE DIAGNOSIS OF LEPTOSPIROSIS

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IT IS recognized that in the majority of cases of human and animal leptospirosis a diagnosis cannot be made without recourse to one or more laboratory procedures.

Leptospirae can be demonstrated by dark-field microscopic examination of the blood during the septicemic stage of the disease, which usually lasts for seven days. Also at this time inoculation of young hamsters with whole blood from patients infected with *Leptospira icterohaemorrhagiae* and *Lept. canicola* generally results in a fatal leptospirosis in the test animal.<sup>1</sup> The leptospirae disappear from the patient's blood stream during the second week and then appear in the urine. Agglutinins, lysins, and complement-fixing antibodies usually begin to appear in the patient's serum toward the end of the first week and are present in readily measurable amounts about the twelfth to fourteenth day after the onset of illness. The maximum titer is reached within three to four weeks and in most instances antibodies are detectable for several months to years.

The tests most frequently used for the demonstration of antibodies in the patient's serum are the macroscopic<sup>2</sup> and microscopic agglutination<sup>3</sup> and agglutination-lysis tests.<sup>4</sup> These procedures require personnel properly trained in the techniques and necessitate the maintenance of suitable cultures of leptospirae. For this reason their use is limited to only a few laboratories.

Because a simple and reliable diagnostic test for leptospirosis was not available, a study was undertaken to develop an antigen for use in a complement fixation test that could be performed in any laboratory conducting the Wassermann or similar complement fixation tests. In the past, the complement fixation test for the diagnosis of leptospirosis has received little attention, apparently because of the difficulty of preparing suitable antigens. Pot and Dornick<sup>5</sup> and Boerner and Lukens<sup>6</sup> have reported on two different methods of preparing antigens for use in the complement fixation test for the diagnosis of Weil's disease. However, Borgen<sup>7</sup> concludes that the lack of agreement between the agglutination reactions and the complement fixation test is due to sources of error in technique or to unstable or inefficient antigens. Where we utilized antigens prepared by present available methods our results also have been unsatisfactory. Therefore, experiments were undertaken to produce a suitable antigen and it was found that leptospirae ruptured by sonic vibration yielded an antigen of considerable specificity and sensitivity.

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## MATERIALS AND METHODS

*Preparation of Antigen.*—Although there are sixteen serologically recognized species of leptospira, only two, *Lept. icterohaemorrhagiae* and *Lept. canicola*, are known to infect human beings and dogs in the United States; therefore, antigens were prepared from these two species. Antigens were prepared from strains of *Lept. icterohaemorrhagiae* and *Lept. canicola* isolated in this laboratory from young hamsters inoculated with whole blood from naturally infected dogs.<sup>1</sup> Erlenmeyer flasks of 250 ml. capacity containing 50 ml. amounts of Stuart's medium<sup>8</sup> were inoculated with 5.0 ml. of a 3-day-old culture of leptospira and incubated at 29 to 30° C. for six days. At the end of this period 0.05 ml. of formalin was added to each flask and the cultures were held at 29 to 30° C. for an additional twenty-four hours. The killed cultures were transferred in 50 ml. amounts to plastic tubes and centrifuged at 17,000 r.p.m. for fifteen minutes in an International Model PR-1 refrigerated centrifuge maintained at 0 to 3° C. The supernatant fluid was decanted and the packed leptospirae were collected and washed three times with 0.85 per cent saline solution buffered at pH 7.0. The washed organisms were then resuspended in buffered saline solution to one-tenth of the original volume of the culture medium. Twenty milliliter aliquots of the concentrated suspension of the leptospirae were exposed to sonic vibration at 9,400 cycles per second for ten minutes in a water-cooled Raytheon Type R-22-3 magnetostriiction oscillator maintained at 100 volts. The material was then pooled and Merthiolate added to 1:1,000 final concentration to constitute the species specific stock antigens. Antigens refrigerated at 4° C. have remained stable and specific for at least six months.

*Complement Fixation Tests.*—The complement fixation technique used at the Army Medical Department Research and Graduate School is employed.

*Complement Titrations.*—The 1:30 dilution of complement is titrated to determine the unit and two full units contained in a 0.5 ml. volume are used for the test. (For example, if hemolysis is just complete in the tube containing 0.14 ml. of complement, then 0.16 ml. is taken as the unit.)

*Antigen Titrations.*—Titrations have shown the optimum dilution of the stock antigens to be 1:15, with the unit ranging from 0.02 to 0.05 ml. of this dilution. Three exact units of antigen contained in a 0.25 ml. volume are employed.

*Inactivation of Sera.*—Human sera are inactivated at 56° C. for twenty minutes, dog and rabbit sera at 62 to 63° C. for forty minutes in a dilution of 1:1. In the routine test, 0.25 ml. of twofold dilutions, from 1:1 through 1:32 and in a quantitative test 1:1 through 1:1,280, of the inactivated serum is used.

*The Test.*—To 0.25 ml. amounts of the sera dilutions three units of antigen and two full units of complement are added. The tubes are refrigerated at 4 to 6° C. for sixteen to eighteen hours. On removal, the tubes are left at room temperature for fifteen to twenty minutes and 0.5 ml. of sensitized cells consisting of 0.25 ml. of a 3 per cent suspension of sheep cells and 0.25 ml. of amboceptor containing three units is added. The tubes are incubated at 38° C. for thirty minutes in the water bath and the test is read.

*Controls.*—The usual hemolytic and positive and negative control sera are set up.

## RESULTS

Parallel complement fixation and microscopic agglutination tests were made on three groups of human sera. Group I consisted of twenty-five random serum specimens submitted to this laboratory from a wide variety of disease processes other than proved cases of Weil's disease. Group II included ten sera from syphilitic patients giving positive Wassermann reactions. Group III included sera from twenty-one proved cases of Weil's disease. In none of the random specimens in Group I were either complement-fixing or agglutinating antibodies against either leptospira antigen detected.

All of the ten positive Wassermann sera of Group II likewise failed to react with leptospira antigens in either type of test.

On the other hand, definite antibody responses were elicited in all of the sera of patients diagnosed as having Weil's disease (Group III). Two examples are shown in Table I.

TABLE I. THE OCCURRENCE OF COMPLEMENT FIXATION AND AGGLUTINATION ANTIBODY IN TWO CASES OF HUMAN LEPTOSPIROSIS

SERUM FROM CASE	DAYS SINCE ONSET OF DISEASE	TITER OF COMPLEMENT FIXATION TEST		TITER OF MICROSCOPIC AGGLUTINATION TEST	
		LEPT. CANICOLA ANTIGEN	LEPT. ICTERO-HAEMORRHAGIAE ANTIGEN	LEPT. CANICOLA ANTIGEN	LEPT. ICTERO-HAEMORRHAGIAE ANTIGEN
1	5	1:8	1:8	0	0
1	28	1:128	1:128	1:10,000	1:1,000
2	6	1:16	1:16	0	0
2	17	1:32	1:32	1:10	1:300
2	42	1:64	1:128	1:100	1:1,000

Case 1 was a patient whose first symptoms of illness were general malaise, headache, conjunctival injection, and pyrexia. Five days after onset, the serum showed a complement fixation titer of 1:8 against both *Lept. canicola* and *Lept. icterohaemorrhagiae* antigens. Another specimen of serum obtained on the twenty-eighth day of illness showed a rise in complement fixation titer to 1:128 against both antigens. The microscopic agglutination test on the first specimen was negative while the second specimen reacted with *Lept. canicola* and *Lept. icterohaemorrhagiae* antigens at 1:10,000 and 1:1,000 dilutions respectively.

Case 2 was that of an attendant in a small animal hospital who developed general malaise, chills, and fever. His serum fixed complement with both leptospira antigens at a dilution of 1:16 on the sixth day of reported illness. By the seventeenth day the titer against both antigens had risen to 1:64 and on the forty-second day the complement fixation titer against *Lept. canicola* was 1:64 and that against *Lept. icterohaemorrhagiae* 1:128. Parallel studies with the microscopic agglutination technique showed negative results on the first specimen of serum, while the second reacted with *Lept. canicola* at 1:10 and *Lept. icterohaemorrhagiae* at 1:300. The third serum specimen had a positive agglutination titer of 1:100 for *Lept. canicola* and 1:1,000 for *Lept. icterohaemorrhagiae*. These observations suggest that antibody formation in leptospirosis may be detectable by the complement fixation test before it is demonstrable by the microscopic agglutination test.

Nineteen of the sera in Group III from human cases of leptospirosis occurring in the Far East were obtained through the courtesy of Dr. Pierre Lepine of the Pasteur Institute. These cases had been diagnosed at point of origin by use of the agglutination-lysis test. Unfortunately, serial specimens were not obtained for comparative studies, but the positive findings obtained on single specimens, as shown in Table II, were in accord with the comparative results of the complement fixation and microscopic agglutination tests noted in Table I.

TABLE II. A COMPARISON OF THE RESULTS OF COMPLEMENT FIXATION AND MICROSCOPIC AGGLUTINATION TESTS WITH SERA FROM HUMAN CASES OF LEPTOSPIROSIS\*

SERUM	DIAGNOSIS	TITER OF COMPLEMENT FIXATION TESTS (SONIC-VIBRATED ANTIGENS)		TITER OF MICROSCOPIC AGGLUTINATION TESTS (LIVE ANTIGENS)	
		LEPT. CANICOLA ANTIGEN	LEPT. ICTERO- HAEMOR- RHAGIAE ANTIGEN	LEPT. CANICOLA ANTIGEN	LEPT. ICTERO- HAEMOR- RHAGIAE ANTIGEN
1	Ictero	1:32	1:32	1:10	1:1,000
2	Ictero	1:16	1:16	0	1:300
3	Ictero	1:32	1:32	1:30	1:100
4	Grippotyphosa				
	Ictero—traces	1:256	1:256	1:30	1:100
5	Ictero	1:32	1:32	0	1:1,000
6	Ictero	1:80	1:80	1:30	1:1,000
7	Ictero	1:640	1:1,280	1:300	1:300
8	Ictero	1:160	1:160	1:30	1:300
9	Ictero	1:80	1:80	1:300	1:1,000
10	Ictero	1:320	1:320	1:300	1:100
11	Grippotyphosa	1:16	1:16	0	1:100
12	Ictero	1:32	1:32	1:10	1:100
13	Ictero	1:64	1:32	1:300	1:3,000
14	Grippotyphosa	1:32	1:32	1:10	1:100
15	Ictero—doubtful	1:64	1:64	1:10	1:10
16	Grippotyphosa—doubtful	1:64	1:64	1:100	1:100
17	Ictero	1:128	1:128	1:30	1:100
18	Ictero—traces	1:128	1:128	1:130	1:100
	Grippotyphosa—traces				
19	Ictero	1:64	1:64	1:10	1:300

Ictero, Lept. icterohaemorrhagiae.

Grippotyphosa, Lept. grippotyphosa.

\*These sera were from cases of leptospirosis occurring in the Far East. They were diagnosed as the result of agglutination-lysis tests. While a diagnosis of the type of infection accompanied each specimen, no history of the cases or titer of the agglutination-lysis tests was available.

Vibrated and nonvibrated fractions of the same lot of leptospirae were titrated for sensitivity against positive leptospiral sera. It was found that the vibrated preparations were approximately three times more sensitive than the nonvibrated preparations.

#### DISCUSSION

Our results indicate that there is a parallelism between the strength of the complement fixing antibody and agglutinins. It should be pointed out that the indicated dilutions of the patient's serum in the complement fixation test should be multiplied by six to be comparable with the serum dilution in the agglutination test.

Although the complement fixation test employing antigens disintegrated by sonic vibrations does not readily differentiate *Lept. canicola* from *Lept. icterohaemorrhagiae* infections, it does demonstrate readily whether or not either of these two types of leptospirosis is present. Sera giving positive reactions to the Wassermann tests and other diverse disease processes fail to react in the same manner as sera from proved cases of leptospirosis. If further differentiation is required, the exact species can be determined by agglutination and absorption tests.

## CONCLUSIONS

A complement fixation test for the diagnosis of leptospirosis (Weil's disease) utilizing antigens obtained from sonic-vibrated leptospirae has been described.

Sera from human cases of leptospirosis caused by *Lept. icterohaemorrhagiae* and *Lept. grippotyphosa* reacted with *Lept. canicola* and *Lept. icterohaemorrhagiae* antigens to titers regarded as specific for leptospirosis.

The specificity of the complement fixation test for the two species of leptospirae occurring in the United States has been demonstrated.

The value of this complement fixation as a simple routine laboratory procedure for the diagnosis of leptospirosis is discussed. It appears that complement-fixing antibody might be detectable before agglutinins could be demonstrated.

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# EXPERIMENTAL VASCULAR DISEASES DUE TO DESOXYCORTICOSTERONE ACETATE AND ANTERIOR PITUITARY EXTRACT

## I. COMPARISON OF FUNCTIONAL CHANGES

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**I**NJECTIONS of crude anterior pituitary preparations elicit in rats morphologic and physiologic changes similar to those caused by desoxycorticosterone acetate (Selye, 1944). Hence Selye (1944) has suggested that the pathogenesis of hypertension, nephrosclerosis, periarteritis nodosa, and certain myocardial lesions can best be explained on the basis of pituitary-adrenal activities. The postulated chain of events would be: stress  $\rightarrow$  anterior pituitary (ACTH)  $\rightarrow$  adrenal cortex (desoxycorticosterone-like compounds)  $\rightarrow$  kidney, blood vessels, heart. The posited identification of the effects of anterior pituitary preparations with those of desoxycorticosterone acetate is lacking in detail. Thus hypertension was estimated either from heart weight (Selye and Stone, 1946) or from single direct measurements of carotid arterial pressure (Dontigny, Hay, Prado, and Selye, 1948).

As part of a more definitive comparison, the present report embodies a survey of the functional effects of anterior pituitary preparations and desoxycorticosterone acetate accomplished by serial measurements of arterial pressure and water diuresis in groups of animals treated with these substances singly or in combination. Diuresis as well as blood pressure was a major criterion in the comparison because of the well-known diuretic effect of desoxycorticosterone. Organ weights also were measured and compared.

## I. EFFECTS OF CRUDE ANTERIOR PITUITARY PREPARATION AND DESOXYCORTICOSTERONE ACETATE

### *Experimental.*—

Sprague-Dawley female albino rats were fed a high protein diet (75 per cent Purina fox chow plus 25 per cent casein) and given 1 per cent saline solution as drinking water. Further "sensitization" (Selye, Stone, Nielsen, and Leblond, 1945) to the hormones was effected by unilateral nephrectomy. Rats were divided into four groups: Group I served as control; Group II received 2.5 mg. of desoxycorticosterone acetate (DOCA) a day; Group III, 30 mg. of a suspension of anterior pituitary powder (APP); Group IV, a combined treatment of DOCA and APP at the same dosage levels as in Groups II and III respectively. Group IV was added in order to see whether the two hormonal preparations would act additively or synergistically.

The steroid was suspended in water in a concentration of 10 mg. per cubic centimeter. The anterior pituitary powder, prepared by lyophilization of cattle glands, was suspended in a concentration of 50 mg. per cubic centimeter in 1 per cent saline containing 10 per cent of

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alcohol and filtered through gauze. The solution was kept in the icebox and made up weekly. Injections were begun immediately after unilateral nephrectomy. The animals were placed in metabolism cages in order to measure diuresis.

Blood pressure was measured by the plethysmographic method of Williams, Harrison, and Grollman (1939) as modified by Kempf and Page (1942). Each value recorded is an average of two to three close consecutive readings. Reproducible results are obtained in nonanesthetized rats under the following conditions. (1) Animals are kept constantly at a temperature of about 30° C.; (2) at the time of the determination, each rat is placed in a constant temperature (40° C.) box for a period of three minutes; and (3) the tail is

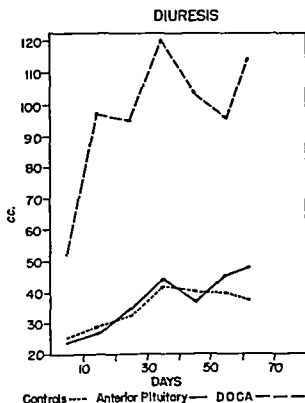


Fig. 1.—Effect of desoxycorticosterone acetate and anterior pituitary preparations on urine formation.

compressed and emptied of blood in the plethysmograph just prior to increasing pressure in the air cuff. From repeated observations on 132 rats, blood pressure was found to vary from 70 to 130 mm. Hg (average 99) with 68 per cent of the animals in the 90 to 110 mm. Hg range. Some of these measurements were compared with those of an electrically recording plethysmograph (Olmsted, Coreoran, Glasser, and Page, 1948) from time to time, as a further check.

Unless otherwise stated, animals were sacrificed on the sixty-first day and autopsied: heart, kidney, ovaries, and adrenals were weighed and sectioned for histologic studies after fixation in Zenker's solution. These detailed histologic observations will be the subject of another paper.

### Results.—

Observations summarized in Figs. 1 and 2 and in Table I are averages of two series of experiments in which the mean initial body weights of the rats were respectively 103 and 219 grams. Results were the same in the two series, so that they are reported jointly. Weights of heart and adrenals are expressed in milligrams per 100 grams of body weight and kidney weights in milligrams per 100 sq. cm. of body surface. The body surface was calculated according to the formula:  $S. A. = Wt. 0.66 \times 11.23$ .

TABLE I. EFFECTS OF DESOXYCORTICOSTERONE ACETATE (DOCA) AND ANTERIOR PITUITARY PREPARATION (APP) ALONE OR IN COMBINATION

GROUP	NUMBER OF RATS	MEAN BODY WEIGHT CHANGES	HEART (MG./100 GM. BODY WEIGHT)	ADRENALS (MG./100 GM. BODY WEIGHT)	KIDNEY (MG./100 CM. <sup>2</sup> BODY SURFACE)
I, Control	21	+43	310 (270-370)	27 (23-30)	315 (286-343)
II, DOCA	20	+17	425 (380-480)	30 (21-37)	513 (426-573)
III, APP	32	+47	402 (250-540)	77 (40-99)	515 (328-675)
IV, DOCA + APP	17	-15	505 (352-608)	64 (38-83)	587 (318-683)

*Growth:* As seen from Table I, all animals gained weight except those of Group IV which received combined treatment with DOCA and APP. In this group, weight loss was noticeable from the beginning of the experiment; the rats became shabby and asthenic, lost interest in food, and began to die around the twenty-first day, so that this experiment had to be terminated on the twenty-ninth day. This effect is surprising because DOCA alone produced a slight inhibition and APP a stimulation of growth. Presumably as a result of anti-hormone formation, the growth hormone effect of APP was much more evident around the twenty-fifth day than on the sixty-first day.

*Diuresis:* Each point on the curves of Fig. 1 represents an average of collections during a period of five days, so that individual variations are eliminated and the curves accurately portray the trend in each group. The increase in diuresis in Group II (DOCA) appears very early and reaches a maximum around the thirty-fifth day. There is no difference in diuresis between control and APP groups; urine formation in both increases slowly but regularly during thirty-five days and then levels off. It is interesting to note that maximum diuresis is reached at the same time as in the DOCA group. Quantitatively, the curve of Group II contrasts markedly with those of Groups I and III.

*Blood Pressure:* Mean blood pressures with individual extremes for Groups I, II, and III are represented graphically in Fig. 2. Blood pressure in the controls was stable during the first six days, after which the trend was variably and slightly upward. The DOCA group showed a definite rise in pressure after the tenth day which reached a maximum at about the twenty-fifth day, at which level it persisted. It is to be noted that *all* the animals in this group became hypertensive. In the APP group, the shape of the curve was more irregular; because of the small proportion of hypertensive animals, it never reached values as high as in the preceding group. Only nine rats out of thirty-two in Group III had pressures higher than 150 mm. Hg. and out of these nine, the two most hypertensive showed pressures of 190 and 195 mm. Hg. respectively. These last two rats died on the fortieth day; this accounts in part for the decrease in pressure mean after this time. In this group, pressures in some animals were always within the normal range. This contrasts with what occurred in the DOCA group.

We have not graphed here the blood pressures of Group IV (DOCA and APP) because this experiment terminated on the twenty-ninth day. Also, the curve did not differ significantly from that of Group II. The mean blood

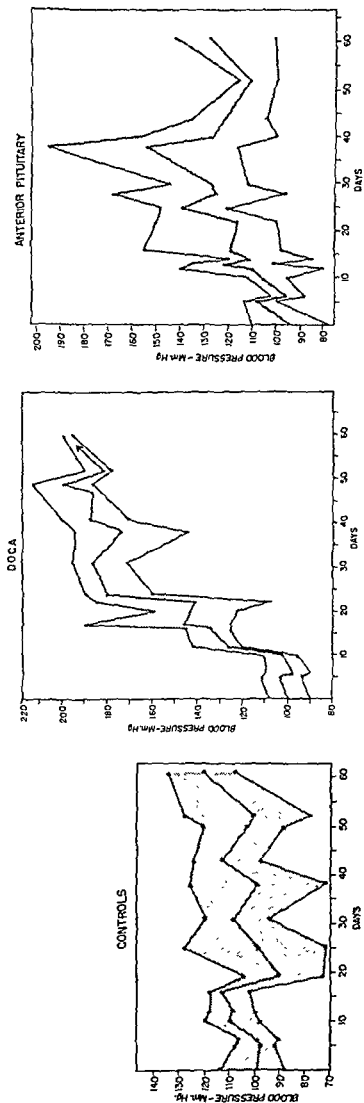


Fig. 2.—Effect of desoxycorticosterone acetate and anterior pituitary preparations on blood pressure.

pressures were: day 0, 93 mm. Hg; day 6, 115; day 12, 140; day 17, 147; and day 29, 161. Thus the combined treatment had no more effect on blood pressure than DOCA alone.

*Organ Weights:* Heart weight was greater in all experimental groups than in the controls, but did not have any consistent relationship to blood pressure levels. Thus mean heart weights in Groups II and III were the same, although the terminal blood pressure averages were, respectively, 190 and 127 mm. Hg. Similarly, kidneys were heavier in the experimental than in the control groups, although growth hormone was administered to Group III only. Adrenal weights were increased in Groups III and IV but unchanged in Group II. The absence of atrophy in Group II was probably due to the low dose of DOCA administered. The adrenal stimulation in Group III is consistent with a relationship between adrenocortical activity and high blood pressure; indeed, it was noticed that the animals presenting the most hypertension were those with the heaviest adrenals. However, closer examination of these glands frequently revealed hemorrhages and necrosis.

#### *Summary.*—

From these experiments we see: (1) with regard to blood pressure, that the effects of DOCA and APP differ significantly in that hypertension is more severe and much more consistent with DOCA than with APP, (2) concerning diuresis, the expected increase is observed with DOCA; administration of APP does not have this effect, (3) that combined treatment with APP and DOCA has no additive or synergistic effect and is in fact rapidly lethal. From this it seems that the mechanisms of the action of DOCA and APP are basically different. However, before concluding, the effects of APP must be examined in more detail.

## II. EFFECTS OF PARTIALLY PURIFIED ANTERIOR PITUITARY EXTRACT

With APP, the maximum rise in mean blood pressure was obtained around the twenty-fifth to the fortieth day and was followed by a decline. Also, a certain degree of resistance and adaptation occurred, evidenced by a decrease in body growth and ovarian weight, toward the end of the experiment. Therefore, it seemed possible that the effects of APP might have been altered by the action of antihormones. To evaluate this possibility, the following experiment was devised in which rats were given progressively increasing doses of pituitary hormones.

#### *Experimental.*—

Female albino rats weighing between 106 and 138 grams (average, 124 grams) were fed a high protein, high sodium diet and were partially nephrectomized. Thirty-six animals were divided equally into three groups: Group I served as control; Group II received subcutaneously the suspension of anterior pituitary powder (APP); Group III received an alkaline anterior pituitary extract (APE). This last group was added in order to test the hypertensive properties of a partially purified anterior pituitary extract, since it has been claimed (Hay and Seguin, 1946) that such preparations cause less kidney damage than crude APP.

This extract was prepared by bringing a water suspension of lyophilized anterior pituitary powder to pH 9 with dilute NaOH. The mixture stood overnight at 10° C.; it was

centrifuged, the precipitate discarded, and the pH of the supernatant adjusted to 7 with dilute acetic acid. The volume of this solution was adjusted so that 1 c.c. contained the equivalent of 80 mg. of the dry powder.

*Animals of Groups II and III* received during the first thirteen days a daily dose of 20 mg. of powder or its equivalent in extract in two injections; the dose was raised to 40 mg. or equivalent until the twenty-third day, and to 60 mg. until the fiftieth day of the experiment, at which time the animals were sacrificed and autopsied.

### *Results.—*

Effects on blood pressure are summarized in Fig. 3. In the control group again, the blood pressure had a tendency to increase slightly during the thirteen days and then to remain relatively stationary. This confirms the observation of Grollman (1946) who noticed a rise in blood pressure following unilateral nephrectomy and administration of NaCl. The blood pressure curves of Groups II and III do not differ significantly; as in the former series with APP, the pressure rose rapidly during the first fifteen to twenty days, then tended to level off. In contrast to our former series with APP, all of the animals showed some increase in pressure, although many were no higher than in the control group. There were few animals with pressures higher than 150 mm. Hg; of these there were two rats in Group II (154 and 176 mm. Hg) and three rats in Group III (150, 185, and 186 mm. Hg).

Organ weights are summarized in Table II. In accordance with the hypothesis that antihormone activity might be overcome by increasing doses of hormones, a sustained stimulation of body growth persisted to the end of the experiment, with no difference between Groups II and III. Heart weights were not significantly different in the three groups. Kidneys were heaviest in Group II and heavier in Group III than in Group I. Stimulation of adrenal growth occurred only in Group II.

TABLE II. COMPARATIVE EFFECTS OF CRUDE (APP) AND PARTIALLY PURIFIED (APE) ANTERIOR PITUITARY PREPARATION

GROUP	BODY WEIGHT		HEART (MG./100 GM. BODY WEIGHT)	ADRENALS (MG./100 GM. BODY WEIGHT)	KIDNEY (MG./100 CM. <sup>2</sup> BODY SURFACE)
	INITIAL	FINAL			
I, Control	127 (120-136)	183 (155-225)	320 (284-405)	23 (20-26)	290 (202-358)
II, APP	123 (106-138)	237 (228-245)	330 (289-428)	33 (26-46)	456 (393-671)
III, APE	124 (118-138)	239 (210-280)	346 (312-428)	25 (18-40)	384 (297-665)

The lack of adrenal stimulation in Group III (APE) can be explained partly by the removal of impurities from the original powder which would act as nonspecific damaging agents in Group II. The difference in the content in impurities between APP and APE was evident at autopsy: abscesses in subcutaneous tissues were present only in APP-treated rats. Further, the relatively low content in adrenocorticotrophin of beef pituitary is well known. The comparative adrenal weights and blood pressures in Groups II and III show a clear dissociation of these functions. Furthermore, increased adrenal weight does not imply increased function, since the adrenals weighing more than 100 mg. were very often hemorrhagic or necrotic.

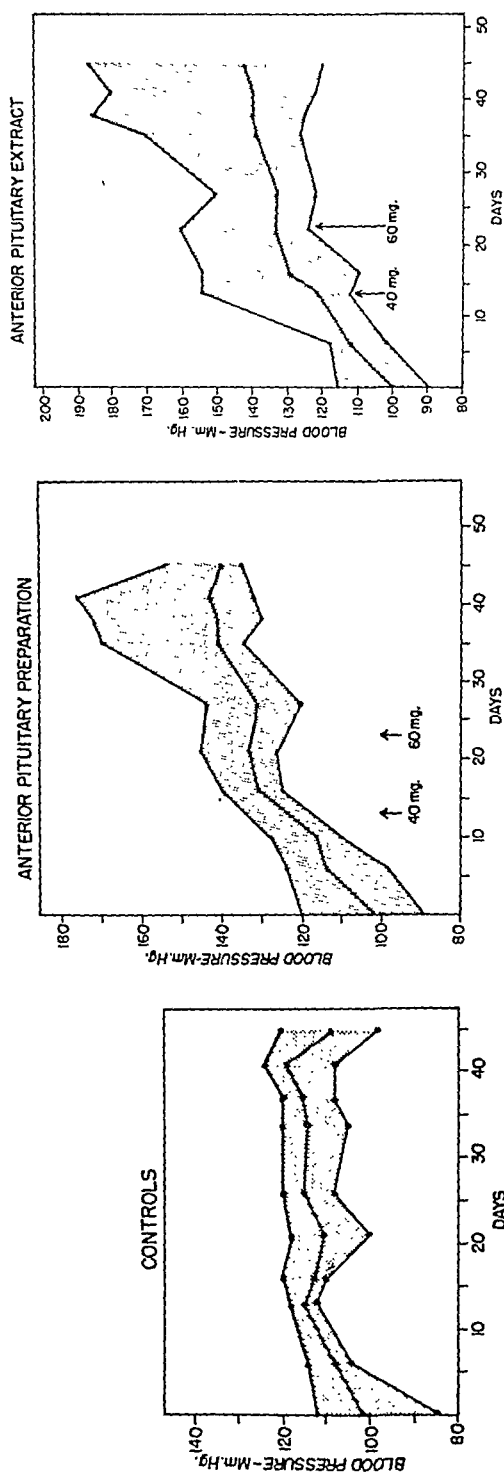


Fig. 3.—Effect of crude and partially purified anterior pituitary preparations on blood pressure.

*Summary.*—

These facts demonstrate a lack of relationship between adrenal growth and blood pressure level which contrasts with observations in animals given crude APP. It follows that the effects on blood pressure of APP may not be wholly the result of adrenal stimulation. Rather, with crude APP the changes in blood pressure and adrenals although concurrent may be unrelated.

## DISCUSSION

Hypertension following DOCA treatment has been observed in rats (Selye, Hall, and Rowley, 1943; Green, 1948; Friedman, Polley, and Friedman, 1948). While Knowlton and co-workers (1946) have reported normal blood pressure in rats treated for ten weeks with 2.5 mg. of DOCA daily and given 0.2 per cent saline solution as drinking water, this apparent inactivity can be attributed to an insufficient supply of sodium. In our experiments, the same amount of DOCA plus 1 per cent saline solution uniformly produced severe hypertension within a period of three weeks. Actually, the amount of DOCA utilized was less than 2.5 mg., for, at autopsy, small deposits of crystalline material were still visible at the sites of injections. Hypertension can therefore be elicited by DOCA as long as the animals are given a high sodium diet.

Injections of anterior pituitary preparations stimulate physiologic body and organ growth when given in conjunction with a normal diet (Selye and associates, 1945); however, pathologic changes (hyalinization and enlargement of glomeruli, hyaline casts, arteriolonecrosis) occur if the animals are partially nephrectomized and maintained on a high protein and sodium diet (Selye, 1944). The latter changes are accompanied by a rise in blood pressure which in our experience seldom reaches the levels obtained following DOCA treatment, experimental perinephritis (Page, 1939), compression of the renal artery (Wilson and Byrom, 1941), or partial renal infarction (Loomis, 1946). Severe pathologic changes and hypertension are seen only in some of the experimental animals, even though large doses are given to all. This is not in accord with the known dose-responses of either steroid or protein hormones. The question arises: Are the pathologic effects of anterior pituitary hormones truly hormonal or do they represent nonspecific effects of foreign proteins or some combination of these actions?

Selye (1944) postulated from observations in rats that anterior pituitary preparations act through the adrenals by releasing DOCA-like compounds. However, a variety of observations made in rats tends to negate this view. (1) Recent studies indicate that it is the zona glomerulosa and not the cortex as a whole which supplies the body with DOCA-like compounds (Sarason, 1943; Greep and Deane, 1947). (2) The zona glomerulosa does not atrophy following hypophysectomy (Smith, 1930). (3) The activity of the glomerulosa depends in part at least on the Na/K ratio of the blood (Deane, Shaw, and Greep, 1948; Nichols, 1948). (4) Anterior pituitary preparations and stress stimulate the inner zone of the adrenocortex which is considered



to be the site of production of glucocorticosteroids (Swann, 1940; Nichols, 1948). (5) There is no proof that glucocorticosteroids have blood pressure raising properties in normal animals. (6) Adrenocorticotrophin and adrenocortical extract tend to decrease blood pressure in hypertensive rats (Corcoran, 1948). Finally, (7) our present observations show a lack of similarity between DOCA- and APP-induced hypertension and a dissociation between adrenal weight and blood pressure level in rats given APE.

Thus we recognize and confirm but cannot fully explain the hypertension caused by DOCA in rats. That this may be a renal hypertension is suggested by its consistent association with renal lesions. We also confirm the fact that hypertension can be produced in rats by injections of APP. However, the view that this hypertension is caused by excessive secretion of DOCA-like compounds from the adrenal is not supported by our data. As to its mechanism, we again point to its association with renal lesions.

The factors which produce these lesions are probably multiple. In the rat, conditions such as high protein diets which greatly increase the renal functional load can result in morphologic lesions (Blatherwick and Medlar, 1937). The renal lesions caused by APP can thus be attributed to the combined effect of a diet rich in protein and sodium, which places a great functional demand on the kidney, and hormonal factors which stimulate renal growth (growth hormone, thyrotrophin, possibly a nephrotrophin\*) (Selye, stone, Nielsen, nad Leblond, 1945; White, Heinbecker, and Rolf, 1949). If it can be accepted that a cell does not function effectively during mitotic fission, it is clear that these superimposed factors will result in a relative renal insufficiency. And, under other conditions of renal insufficiency, such as subtotal nephrectomy (Chanutin and Ludwig, 1939; Addis, Barrett, Lew, Poo, and Yuen, 1946) or serum nephritis (Smadel and Farr, 1939), high protein diets are known rapidly to precipitate morphologic regression. Another mechanism of renal damage may be the foreign proteins contained in the pituitary preparations, since injections of proteins are known to cause degenerative renal lesions in rodents (Masugi and Sato, 1934; Oberling, 1943).

#### SUMMARY AND CONCLUSIONS

1. Desoxycorticosterone acetate (DOCA) and anterior pituitary preparation (APP) were tested in unilaterally nephrectomized female rats fed a high protein and a high sodium diet.

2. DOCA (2.5 mg. a day) elicited hypertension in all animals; APP had this effect in only a relatively small proportion of animals tested. Combined treatment with DOCA and APP had no more effect on blood pressure than DOCA alone; rather, the animals rapidly sickened and died.

3. DOCA caused severe diuresis, while control and APP-treated groups showed only a slight increase in urine formation at the beginning of the experiment.

\*We suggest this term as etymologically preferable to the term "renotrophin."

4. In rats treated with crude APP there is an association between hypertension and increased adrenal weights. But these large adrenals are often hemorrhagic or necrotic. The relationship of adrenal weight to blood pressure disappears when animals are treated with a partially purified APP extract.

5. The hypothesis that APP hypertension in rats depends on hypersecretion of DOCA-like compounds thus appears unlikely.

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# RETROGRESSION OF ATHEROSCLEROTIC LESIONS ON CESSATION OF CHOLESTEROL FEEDING IN THE CHICK

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**D**ESPITE the multitude of experiments in which cholesterol has been fed to many species in order to induce atherosclerosis, little is actually known concerning the natural history of the lesions so produced. The observations recorded in the literature are few and fragmentary,<sup>1-5</sup> based on small groups and in some instances on individual animals. In view of the large volume of work in the field of experimental atherosclerosis, the need for a standard base line for comparison is imperative. With this end in view, we have already reported on the relationship of atheromatosis to the amount of cholesterol added to the diet in the chicken.<sup>6</sup> We demonstrated a semiquantitative relationship between the per cent of cholesterol in the diet and the duration of feeding, on the one hand, and the severity of the resultant atheromatosis on the other. It was found that for concentrations of dietary cholesterol above  $\frac{1}{2}$  per cent there was no increase in the degree of atherosclerosis upon prolonging the feeding period from ten to fifteen weeks. We therefore decided to investigate the effects of cessation of cholesterol feeding on the hypercholesterolemia and atherosclerosis resulting from moderate periods of cholesterol feeding.

## METHODS

One hundred and fifty-five 6- to 8-week-old white leghorn cockerels were placed on a diet of chick starter mash enriched with 2 per cent cholesterol in 20 per cent cottonseed oil and fed ad libitum. In our experience, when given for ten weeks this diet produces atherosclerosis in 100 per cent of chicks.<sup>6</sup>

After ten weeks of feeding, the surviving cholesterol-fed chicks were subdivided into three groups. Thirty-one chicks continued to receive cholesterol for an additional fourteen weeks. Twenty-five were placed on a diet of chick starter mash, and the third group of twenty-five received specially prepared mash† from which the fat and cholesterol had been extracted by a commercial degreasing process. By this means the fat content of the mash was reduced from 4.5 per cent to 0.2 per cent, and the cholesterol from approximately 100 mg. per cent to 0. Vitamin supplements and sucrose were added to make this diet equivalent in caloric value to normal mash.<sup>7</sup> Twenty-six control chicks were maintained on commercial chick starter mash throughout the entire experimental period.

Chicks were sacrificed at the conclusion of the ten-week period of cholesterol feeding and at intervals of three to four weeks thereafter. Because of spontaneous deaths among the

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†Generously supplied by The Armour Laboratories, Chicago, Ill

flock, it was not possible to adhere to a rigid schedule of sacrificing. However, attempts were made to obtain comparable samples from all the groups. All birds were autopsied, the organs inspected and the hearts and aortas dissected out en bloc, opened, and graded for extent and severity of atherosclerosis. A sketch of the lesions was made on a specially prepared form, and they were graded 0 to 4 on the basis of criteria in use in our laboratory.<sup>6</sup> The thoracic and abdominal portions of the aorta were graded separately but have been combined in our tables for simplicity in presentation. Tissues were preserved in formalin. Sections were taken from the most severely involved areas in the aorta of each bird, and wherever possible specimens were taken from both the thoracic and the abdominal aorta. Hematoxylin and eosin stained specimens and frozen sections stained with sudan IV were prepared and studied.

Blood was drawn from the alar vein for cholesterol determination by the method of Schoenheimer and Sperry.<sup>8</sup> Samples were taken at five and ten weeks after commencement of the experiment, and then at weekly and biweekly intervals until the conclusion of the experiment.

## RESULTS

*A. Morphologic Observations in the Various Groups.*—It seemed a worthwhile procedure to discuss the morphology in some detail because (a) this is the longest period of cholesterol feeding undertaken in the chick, along with an equally adequate group of controls for comparison, and (b) this is the first time, in a species other than the rabbit, that the opportunity has been afforded for observations over an extended period on the effects of cessation of cholesterol feeding on the arterial intima of the chick.

*Controls Receiving No Cholesterol Throughout Study:* Of the four control chicks which showed gross lesions, one had a lesion of the thoracic aorta alone, two had lesions of the abdominal aorta, and one had lesions in both portions of the aorta. Of the gross lesions, those in the thoracic aorta were flat, nonraised, whitish or light-yellow areas, varying in size from a few millimeters to 1 cm. in their greatest dimension. In the abdominal aorta the lesions are seen in a prominent ridge-like area lying between the renal arteries on the posterior wall of the aorta. They are characterized by a light-yellow staining of this area. Microscopic lesions were far more common. Nine out of twelve aortas from which frozen sections were made showed evidences of spontaneous atherosclerosis. The lesions have already been described fully by Dauber<sup>9</sup> and Chaikoff and associates,<sup>10</sup> and we are in full accord with their findings. Typical lipid deposits were found in the thoracic aorta in our cockerels. Microscopically the hematoxylin and eosin sections of thoracic aorta showed only scattered focal areas of loosely arranged ground substance which had almost the appearance of mucoid degeneration. These foci lay between the elastic and collagenous fibers of the intima and between the elastic laminae of the media. On frozen section, these areas were strongly sudanophile, and the stained material lay free in the ground substance (Fig. 1, A). Chaikoff and co-workers<sup>10</sup> have examined these deposits under polarized light and believe them to be free of cholesterol, containing only fat. Fatty material was most commonly found in the inner third of the media. The myocardium and coronary arteries were not involved in any way. The most striking changes were seen in the abdominal or muscular section of the aorta. As described by Dauber,<sup>9</sup> the primary lesions were to be found in the ridge-like prominence in the interrenal region. The lesions are characteristically fibrotic, being made up of collections of collagenous bundles and fibroblasts, with small to moderate amounts of sudanophile material in the depths of the plaques, usually next to the intimal-medial boundary. An excellent illustration of such a lesion is Fig. 1, B.

*Birds Maintained on 2 Per Cent Cholesterol Diet Throughout:* The earliest gross lesions were observed in the brachiocephalic vessels and the elastic aorta (referred to as the thoracic aorta). They appeared in the form of flat, nonraised, white or whitish-yellow areas never

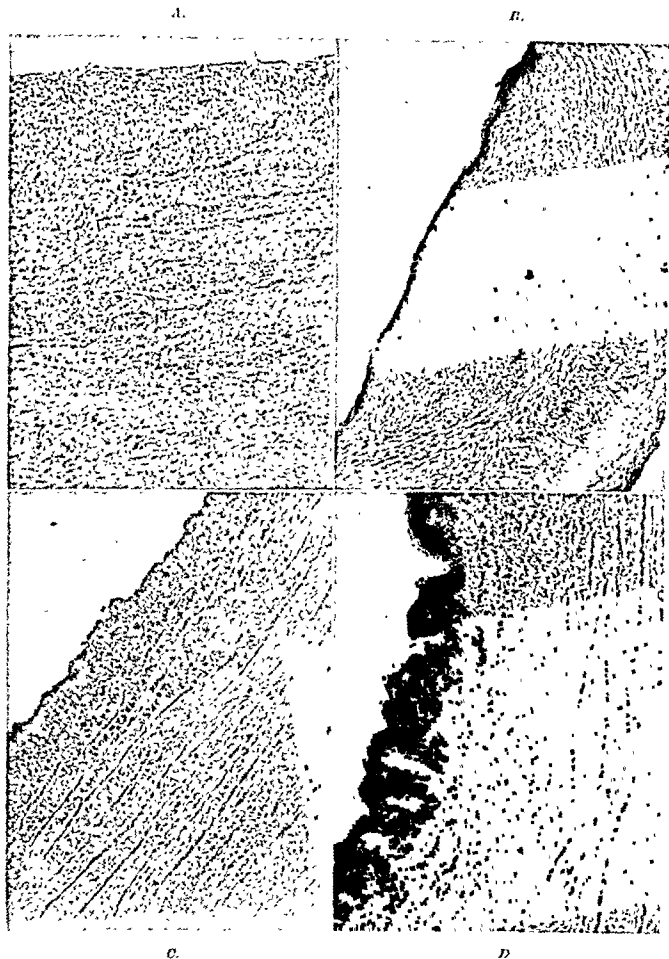


Fig 1—A, Bird 23, control, on normal mash diet twenty-four weeks. Spontaneous lesion of the thoracic aorta. There is focal accumulation of sudanophile material in the intima and media (note dark-staining areas). (Frozen section,  $\times 120$ , sudan IV.) B, Bird 37, control, on normal mash diet fourteen weeks. Spontaneous lesion of the abdominal aorta. There is marked fibrous and fibroblastic thickening of the intima. Region near intimal-medial boundary contains small amount of sudanophile material. (Frozen section,  $\times 120$ , sudan IV.) C, Bird 167, on cholesterol fourteen weeks. Early cholesterol-induced lesion of the thoracic aorta. Note deposition of sudanophile material in intima and inner portion of media. (Frozen section,  $\times 120$ , sudan IV.) D, Bird 191, on cholesterol twenty-four weeks. Very heavy deposit of sudanophile material in the intima and inner one-third media. (Frozen section,  $\times 120$ , sudan IV.)

more than a few millimeters in diameter. This progressed to increasing thickening of the intima in these regions and the appearance of more extensive, more deeply pigmented patches, which were striated or nodular in gross appearance. Progression led to severe involvement of almost the entire elastic aorta by either a confluent plaque or separate and extensive nodular or granular plaques. There was a clear line of demarcation at the junction of the elastic and muscular aorta. The aortic valves were frequently involved with heavy plaques of yellow material in the sinuses of Valsalva. The mitral valves showed pin-point lipid deposits in their substance. We again noted<sup>6</sup> that there is a definite time lag in the involvement of the abdominal portion of the aorta. The interrenal area is preferentially involved. Involvement ranged from slight yellow streaking to almost complete occlusion of the lumen of the aorta by massive deep-yellow plugs. Isolated nodular involvement of the proximal portion of the iliac vessels was a common phenomenon.

Microscopically, the least severe lesions consisted merely of some increased deposition of gray-blue ground substance between the collagenous and reticular fibers of the intima. On frozen section and sudan staining these areas contained moderate amounts of sudanophile material and an increased number of fibroblasts (Fig. 1, C). Sudanophile material could also be seen scattered lightly through the inner half of the media. Most of the early lesions however consisted of foam cell plaques of varying degrees of severity—from a single layer of cells to veritable foam cell cushions, several layers deep. The fat-filled fibroblastic cells were arrayed along vertically disposed reticular fibers and their nuclei also were perpendicular to the intimal coat. Usually foam cells and an increased amount of intercellular ground substance could be seen infiltrating the inner half to one third of the medial coat (Fig. 1, D). The coronary arteries were relatively uninvolved in these early lesions. As the period of cholesterol feeding was prolonged, more severe changes were seen in the thoracic aorta. The progression is not clear in all instances, i.e., some lesions were seen at 140 days which, comparatively speaking, were no more severe than those seen at 70 days. However, a number of very severe lesions were seen late in the feeding period. Hyaline and cartilaginous metaplasia, associated with breakdown of the foam cell plaques, appears to be the first step. With time there is a disappearance of the well-formed foam cells, although ghost cells can be seen in the areas undergoing hyaline metaplasia. Typical cartilage does appear in the mid-depth of the plaque, which eventually becomes heavily basophilic and in some instances undergoes extensive calcification. In some sections there is actual breakdown of the centers of the foam cell plaques with the formation of "abscesses" containing necrotic debris, fragments of nuclei, free fat, cholesterol crystal clefts, and heavy deposits of calcium in granules or plates. In the heavier foam cell plaques which remain intact, numerous fine vasa may be seen communicating with the lumen, and endothelialized spaces were found resembling vasa which are filled with foam cells, loose and in clumps (Fig. 2, A).

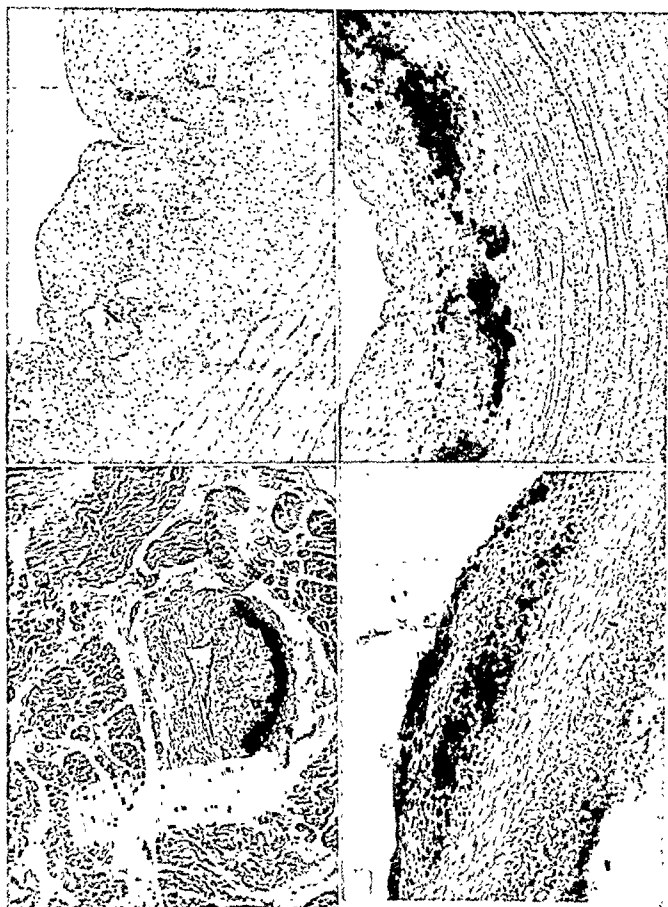
The media is involved in all instances, particularly in its inner half, by infiltration of foam cells and free fat. Where the intimal lesions are very severe, there may be severe attenuation of the underlying media. Involvement of the coronary arteries is common in association with severe aortic atheromatosis. Foam cell plaques may be seen partially or completely occluding the lumina of small arterioles. Some of the lesions also show associated calcium granule deposition. Fig. 2, C shows a coronary arteriole severely narrowed by foam cells with a heavy plaque of calcium almost completely replacing the underlying media.

The thoracic aorta therefore shows a spectrum of changes extending from minimal deposits of fat and cholesterol in the ground substance to extensive foam cell plaques, atheromatous abscesses, hyalinization, cartilaginous metaplasia, and calcification.

There appeared to be little or no correlation between the severity of the lesions in the thoracic aorta and those in the abdominal aorta. Early lesions in the abdominal aorta were of two types. One group was indistinguishable from those of the "spontaneous" type which

A.

B.



C.

D.

Fig. 2—*A*, Bird 188, on cholesterol fifteen and one-half weeks. Severe foam cell plaque of thoracic aorta. Note vasa vasorum containing red blood cells, and also the endothelialized spaces filled with foam cells, discrete and in clumps. ( $\times 120$ , hematoxylin and eosin.) *B*, Bird 169, on cholesterol thirteen and one-half weeks. Advanced lesion of thoracic aorta. Note superficial layer of dense connective tissue, beneath which is a wide zone of cartilaginous metaplasia with numerous foci of calcification. Involves inner one-third of media as well as intima. ( $\times 120$ , hematoxylin and eosin.) *C*, Bird 479, on cholesterol seventeen weeks. Severe narrowing of lumen of coronary artery by foam cells. There is a heavy plate of calcium almost completely replacing the underlying media. ( $\times 120$ , hematoxylin and eosin.) *D*, Bird 191, on cholesterol twenty-four weeks. Lesion of abdominal aorta shows superficial layer of dense fibrosis with much lipid and "abscess formation" in the depths of the plaque. (Frozen section,  $\times 120$ , sudan IV.)



were seen in the controls, i.e., they were characterized by fibrotic changes with small amounts of sudanophile material and calcium granules in the very depths of the plaques along the intimal medial boundary. A second group likewise showed severe fibrotic changes, but was characterized by the presence of a considerable amount of lipid and cholesterol crystals and calcium throughout the plaque. Small atheromatous "abscesses" may be seen lying deep beneath a fairly heavy collagenous and fibroblastic coat (Fig. 2, D). With progression of the cholesterol feeding, the latter type of lesion predominates and becomes more severe. A third type of lesion becomes apparent as the period of feeding is prolonged. This lesion is very extensive both vertically and horizontally—it is several times the thickness of the uninvolved vessel wall. The surface of this lesion is coated by a fine layer of collagenous fibers, with few fibroblastic nuclei. Beneath this lies a deep layer of structureless gray-blue ground substance containing some foam cell ghosts, numerous cholesterol crystal clefts, and many calcific granules. The homogenous ground substance is usually intersected by fine strands of collagenous fibers, giving the impression of periods of accretion and quiescence. The deepest layers show an increased number of cholesterol crystal clefts and heavier deposits of calcium both in granules and in plaques. The media is partially or completely obliterated and replaced by a thin layer of fibrous connective tissue (Fig. 3, A).

It seems, therefore, that the lesions produced by cholesterol feeding in the abdominal aorta are superimposed on and/or accelerate the evolution of the so-called "spontaneous" lesion. The presence of dense fibrosis in the less severe lesions and the relative paucity of fat are probably a manifestation of the body's defense against the deposition of cholesterol and fat. When this resistance is overwhelmed, one probably sees the massive structureless lesions described.

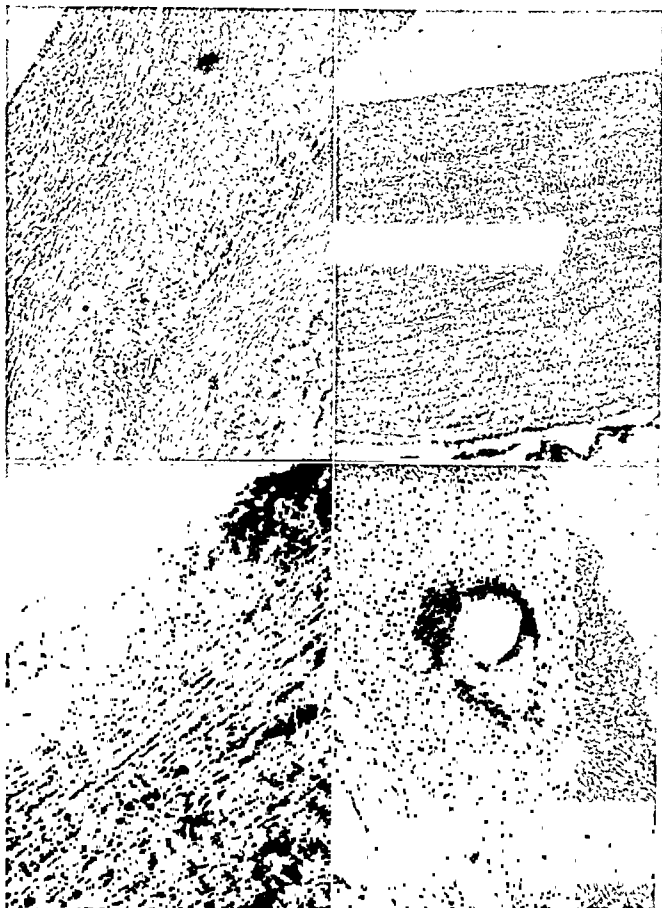
*Birds on Cholesterol for Ten Weeks and Then Placed on Low Fat or Ordinary Mash Diet:* Grossly and microscopically the group in which cholesterol was replaced by ordinary mash was essentially similar to the group placed on the low fat diet.

Grossly, the lesions seen in this combined group differed little in point of appearance from those seen in the group maintained on cholesterol throughout. However, they tended to be less yellow and to have a whitish tinge. They were definitely less extensive and less severe. Both the thoracic aorta and the abdominal aorta appeared dull-white and thickened in a number of instances, but showed little or no involvement with atheroma. Microscopically, up to seven weeks the lesions were not markedly different from those seen in cholesterol-fed birds sacrificed at the time of division or shortly thereafter. There was moderate thickening of the intima by an increase in ground substance, and there was cellular and foam cell proliferation. The lesions of the abdominal aorta were principally fibrocellular with deeper portions of the plaques containing abundant fat, cholesterol crystals, and fine particles of calcium. From seventeen to twenty-four weeks the severity of the intimal lesions was less—fibrocellular thickening of the intima was more common and foam cells were less frequently seen. However, occasional lesions persisted which were quite severe. One chick showed a heavy foam cell plaque at seventeen weeks; another showed hyaline metaplasia at nineteen weeks, and a third showed heavy calcification at nineteen weeks. On the whole, however, intimal thickening in the thoracic aorta, when present, was principally fibrocellular in character (Fig. 3, B). An interesting phenomenon was observed in those chickens which had been longest on the low fat diet (i.e., had had the longest respite from cholesterol feeding). Lying directly beneath the slightly fibrosed intima were large globular cells filled with fat. They could be made out individually and seemed to be migrating toward the lumen (Fig. 3, C), as if they were scavengers carrying fat and debris out of the lesion into the lumen of the vessel.

The abdominal aorta was characterized by two types of lesions: (1) a predominantly fibrotic lesion probably of the so-called spontaneous variety with little sudanophile material,

A.

B.



C.

D.

Fig. 3.—A, Bird 488, on cholesterol for fifteen weeks. Very severe lesion of abdominal aorta. Surface of lesion is coated by a thin layer of connective tissue, beneath which lies a deep layer of ground substance intersected by fine collagenous strands and containing cholesterol crystals, calcium granules, and foam cell ghosts. ( $\times 120$ , hematoxylin and eosin.) B, Bird 63, on cholesterol for ten weeks and then on ordinary mash for fourteen weeks. There is very minimal thickening of the intima of the thoracic aorta which is fibrocellular in character. ( $\times 120$ , hematoxylin and eosin.) C, Bird 97, on cholesterol for ten weeks, then on ordinary mash for fourteen weeks. Note globular sudanophile masses immediately beneath endothelial lining of vessel. Also scattered sudanophile granules in the remainder of the intima. (Frozen section  $\times 480$ , sudan IV.) D, Bird 147, on cholesterol for ten weeks, then on special low fat diet for five weeks. Note atheromatous "abscess" cavity surrounded by fibrocellular connective tissue. (Frozen section,  $\times 120$ , sudan IV.)

and (2) more severe lesions showing heavy fibrosis but also severe residual deposits of sudanophile material and calcium. The latter probably represent the evolution of the second type of early abdominal lesion seen in the continuing cholesterol feeding. Fig. 3, *D* is an illustration of one of these. It shows marked thickening of the intimal surface with fibrocellular connective tissue and partial obliteration by fibrous tissue of an atheromatous abscess. The cavities contain sudanophile material and heavy deposits of calcium. The underlying media shows some fibrosis.

In summary, cessation of cholesterol feeding and the institution of a low fat or normal mash diet were followed by fibrotic changes in lesions of both the thoracic and abdominal portions of the aorta, by the disappearance of foam cells, and by calcification of atheromatous abscesses. We also have noted the presence of scavenger-like cells in the intima.

*B. Summary of Data on Degree of Atherosclerotic Lesions in Various Groups.*—Table I summarizes the average gross gradings in the various groups for the entire duration of the experiment. The base line value of the severity of lesions at the end of ten weeks of feeding with cholesterol is based upon all chicks, both experimental and control, which either died or were sacrificed. Also included in this base line group are three birds from the low fat mash group and two from the plain mash group which died during the first week following division of the groups. The average gross grading for the eighteen chickens in this group was 2.5, which compares favorably with values reported by us in a previous experiment.<sup>6</sup>

TABLE I. AVERAGE GROSS GRADING OF AORTIC ATHEROSCLEROSIS IN ALL GROUPS FOR ENTIRE DURATION OF EXPERIMENT

TIME FROM BEGINNING OF EXPERIMENT (WK.)	ON 2% CHOLESTEROL DIET FOR 10 WEEKS AND THEN CONTINUED ON						CONTROLS ON PLAIN MASH	
	2% CHOLESTEROL		LOW FAT DIET		PLAIN MASH		NUMBER OF BIRDS	AVERAGE GRADE
	NUMBER OF BIRDS	AVERAGE GRADE	NUMBER OF BIRDS	AVERAGE GRADE	NUMBER OF BIRDS	AVERAGE GRADE		
9 to 11	18*	2.5					2	0
12 to 14	6	4.1	6	2.7	12	2.4	5	0.05
15 to 18	11	4.3	4	2.1	4	1.8	5	0.05
19 to 22	4	4.3	7	1.1	3	3.5	2	0
22 to 24	4	5.9	5	0.5	4	1.0	12	0.1
Total	43		22		23		26	

\*Includes seven chicks which died in the week prior to separation of the groups and three from the low fat and two from the plain mash groups which died during the first week after separation of the groups.

*Continued Cholesterol Feeding:* This is best visualized in Fig. 4. The average gross grade rose from 2.5 at the time of division (ten weeks) to 4.1 for the periods fifteen to twenty-two weeks, and then rose to 5.9 for the period twenty-three to twenty-four weeks. There was, therefore, an early rapid rise in severity of lesions, then a tendency to level off after fifteen weeks of cholesterol feeding, and finally a further rise after twenty-two weeks of cholesterol feeding.

*Normal Mash:* Placing cholesterol-fed birds on a diet of normal mash resulted in a gradual decline in the extent and severity of the atherosclerosis.

This is best seen for the periods fifteen to eighteen weeks and twenty-three to twenty-four weeks, where the average values obtained were 1.8 and 1 respectively. For the period eighteen to twenty-two weeks a value of 3.5 was obtained. This average was constructed from data from three chicks, one of which had very severe lesions. On the whole, however, the trend toward decreasing severity of the lesions was unmistakable.

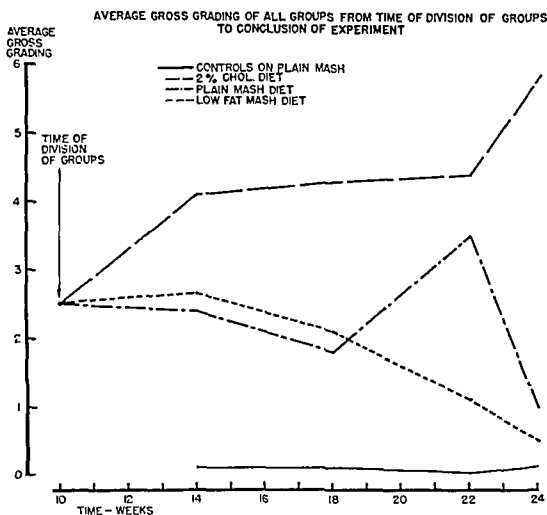


Fig. 4.—Average gross grading of all groups from the time of division into groups to the conclusion of the experiment.

*Low Fat:* The group of cholesterol-fed birds placed on a low fat, cholesterol-free diet gave values which closely resembled those obtained in the group of cholesterol-fed birds placed on ordinary mash, but the decline in the severity of the lesions was somewhat more marked and was consistent for all time periods. The decline first became evident at the fifteen to eighteen weeks period and was most noticeable during the eighteen to twenty-two and twenty-three to twenty-four week periods, where average gross values of 1.1 and 0.5 were recorded.

Combining the low fat and normal mash groups gave us a more adequate set of averages. Again a progressive decline in the severity of the lesions was apparent.

*Controls:* The control group which received normal mash with no added cholesterol over the entire period was remarkable for the paucity of grossly

discernible atheromatous lesions of the so-called "spontaneous" variety. Only four of the twenty-six controls showed grossly visible atherosclerosis. Two were graded at  $\frac{1}{4}$ , one at  $\frac{1}{2}$ , and one at 1.

*C. Blood Cholesterol Levels.*—Fig. 5 illustrates the blood cholesterol levels for the various groups throughout the course of the experiment. The first and second determinations were made at five and ten weeks after commencement of the experiment. Thereafter blood cholesterol determinations were

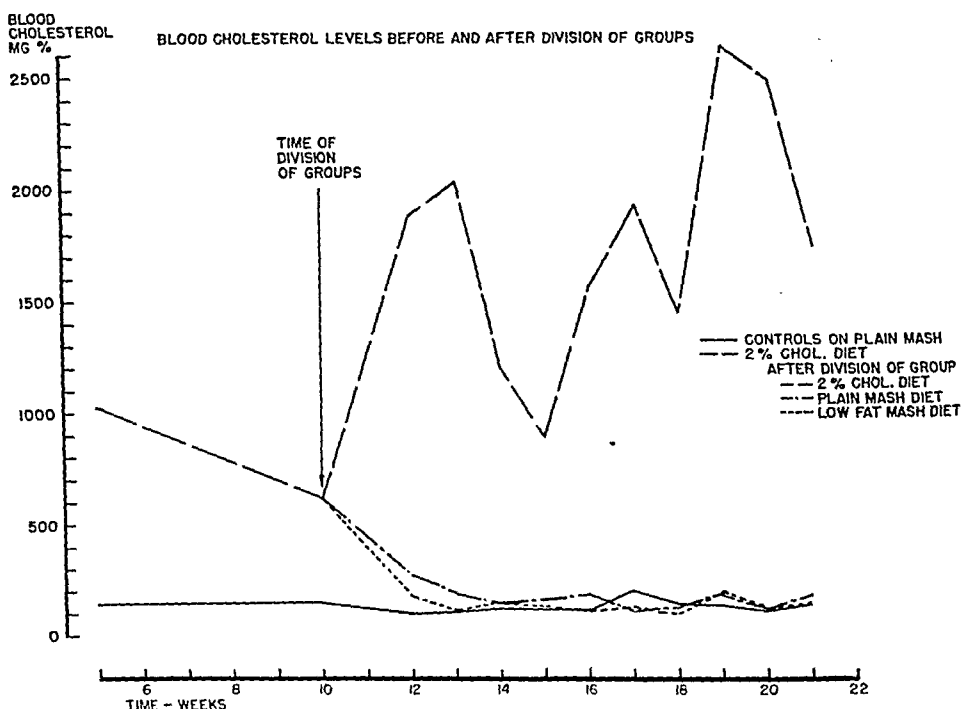


Fig. 5.—Average blood cholesterol levels before and after division of birds into the several groups.

made at weekly or biweekly periods. The values for the 2 per cent cholesterol-fed birds showed a good deal of variation from week to week, but the trend on the whole was toward increasing levels. The last three determinations were 2,629, 2,479, and 1,839 mg. per cent respectively. During the same period the blood cholesterol level fell in the groups taken off the cholesterol diet. Within two weeks of cessation of cholesterol feeding, the blood cholesterol levels of the plain mash and low fat groups fell to 268 and 175 mg. per cent respectively (control, 100 mg. per cent). Within one more week the values for both groups were back in the normal range. The blood cholesterol levels of the controls showed no significant variation during this period.

In summary, continued cholesterol feeding after ten weeks results in the maintenance and further elevation of the blood cholesterol levels, while cessa-

tion of cholesterol feeding leads to a very rapid fall of the blood cholesterol back to normal levels.

#### DISCUSSION

The results obtained in the present study complement and extend our previous studies on cholesterol-induced atherosclerosis in the chicken. It is now clear that continuing cholesterol feeding in the chick for periods up to six months results in progressive aggravation of the atherosclerosis. This is not in accord with previous data reported by us for a much shorter experimental period.<sup>6</sup> The progression is associated with a continued rise in the blood cholesterol levels. The spectrum of microscopic findings resulting from continued cholesterol feeding is wide indeed, ranging from mere increases in amorphous ground substance to massive foam cell plaques, hyaline and cartilaginous metaplasia, and heavy calcium deposits. We noted also the tendency for cholesterol-induced lesions of the abdominal aorta to appear in an area usually involved in the "spontaneous" variety of the disease and apparently to accelerate and/or aggravate the normally occurring lesion.

Cessation of cholesterol feeding after ten weeks resulted in a gradual decline in the severity of the lesions as judged by gross grading in both the plain mash and low fat groups. There was no essential difference between the two groups either from a gross or microscopic standpoint, and they have been combined for purposes of discussion (and in Table I). Blood cholesterol levels fell to normal within three weeks of cessation of cholesterol feeding. Microscopically the lesions were characterized by diminution and disappearance of foam cell deposits, increasing fibrotic changes in both the abdominal and thoracic portions of the aorta, diminution of grossly stainable lipid, and heavier calcification. Some of the chickens sacrificed at the very end of the experiment showed so little gross or microscopic evidence of atherosclerosis that it may be assumed that the lesions in these birds had undergone complete remission. In other birds, severe fibrosis, remains of atheromatous abscesses, and heavy calcification tended to suggest that there is a limit to the degree of atherosclerotic damage which can be repaired.

Controls kept on ordinary mash showed only few gross lesions, but the incidence of microscopic lesions was much higher. This is in accord with previous experience in this laboratory.<sup>6</sup> In corroboration of Chaikoff and co-workers,<sup>10</sup> we found lipid deposits in the thoracic aortas of cockerels, a phenomenon reported by Dauber<sup>9</sup> only in hens. These lesions of the thoracic aorta are never characterized by thickening of the intima or by foam cell deposits, but consist of small focal deposits of lipid material in the ground substance of the intima and inner portion of the media. In the abdominal aorta, the spontaneous lesions are characteristically fibrotic with far less stainable lipid than in the cholesterol-induced lesions. They are, however, very similar to some of the less severe lesions induced by cholesterol feeding, particularly when the latter has been suspended for some time and the chicks have been returned to a normal diet.

Other investigators have made observations on the effect of suspending cholesterol feeding on the arterial lesions of the rabbit. Stuckey,<sup>1</sup> Krylow,<sup>2</sup> Wada,<sup>3</sup> and Scarff<sup>4</sup> were the first to report such observations, but their data is not very extensive. Anitschkow<sup>5</sup> was the first to study the problem extensively in the rabbit. He found that following three to four months of cholesterol feeding, and the resumption of a normal diet, there resulted a gradual loss of lipids from the plaques. The latter lost their yellowish color and no longer stained with sudan. They presented a whitish and fibrous appearance. He stressed, however, that this process was a slow one. The transformation of a large plaque rich in lipids into a plaque composed of fibrous tissue took two to three years, and even then small quantities of lipid could be discovered microscopically after sudan staining. Microscopically Anitschkow noted the gradual disintegration and disappearance of the foam cell masses and their replacement by newly developing collagenous and elastic fibers and fibroblasts. He noted also the tendency for cholesterol crystals to persist long after the other lipids have been removed, and also the large quantities of calcium which were laid down in areas formerly occupied by atheromatous abscesses. He stated that if the development of the lipid deposits and the thickening of the intima had not reached a very considerable degree during the progressive period, all that was to be seen after resorption of the lipids had taken place was a slight fibrous thickening of the intima, sometimes containing a few cholesterol crystals, together with scattered lipid cells and globules of neutral fat. Anitschkow gave no data on the effect of cessation of cholesterol feeding on the blood cholesterol levels in the rabbit. Steiner,<sup>12</sup> among others, has observed that following the cessation of cholesterol feeding in rabbits the blood cholesterol levels fall very slowly over a period of months. It is of interest that the resorptive changes seen in the rabbit, and described by Anitschkow and others, so closely resemble the changes seen by us in the chicken. It is also of interest that the rate of regression of lesions in the chicken is much greater than in the rabbit. These two features may well be related in an etiologic and pathogenetic sense, as will be discussed.

The ability of the physiologic mechanisms to remove lipids from the arterial wall is not a feature common to the experimental animal alone. In man, Leary's<sup>13</sup> excellent studies have thrown considerable light on the problem of the bodily defenses against the development of atheromatous lesions. Leary believes that a defense mechanism exists which removes excess fat and cholesterol from the arteries of youth and from the ascending aorta even in old age. The cholesterol is transferred from wandering lipid cells (foam cells) to fixed fibroblasts in which the cholesterol esters are split, anisotropism is lost, and the cholesterol is brought into solution in an excess of fatty acids. Solution is followed by its disappearance from the lesions. With the removal of the excess cholesterol, the lesions stop progressing toward atherosclerosis and subside. In youth this mechanism is adequate to cope with lipid deposits in the intima. As man ages his ability to remove cholesterol appears to diminish, and atherosclerosis becomes severe. Increased deposition of collagen

and dense scar tissue with subjacent collection of fat is more common. In old age, cholesterol removal mechanisms appear to cease, and foam cells accumulate in masses, with inadequate nutrition and support, and primary atheromatous abscess is the typical lesion. Further corroborative evidence on the resorbability of human lesions comes from two sources. Aschoff<sup>14</sup> observed that atherosclerotic lesions were rare in autopsy material during and after World War I in Germany—a period of severe famine and malnutrition, especially with respect to fats, cholesterol, and proteins. More recently Wilens<sup>15</sup> has noted that at necropsy severe atherosclerosis is at least twice as common in obese as in undernourished persons 35 years of age or over. He found this relationship to be independent of sex or the presence or absence of diabetes or hypertension. Severe or even moderate atherosclerosis was seldom found in those with protracted undernutrition. Less severe atherosclerosis was observed in those with wasting disease than in those with terminal weight loss. He inferred from this data that resorption of atheromatous lesions may occur during periods of marked weight loss. Histologically the most conspicuous difference between the two groups lay in the amount of lipid in the intimal lesions—there were less lipids and fewer phagocytes in the intimal lesions of those with weight loss.

Thus far we have discussed only the spontaneous regression of lesions which takes place in the experimental animal on the cessation of cholesterol feeding and in man following prolonged undernutrition. We have already noted that the provision of a low fat, cholesterol-free diet did not appreciably affect the rate of regression of lesions in the chick. Attempts have been made to influence the rate of regression of lesions in the rabbit through the use of various substances known to influence fat metabolism. Steiner<sup>12</sup> noted that the administration of choline to rabbits which had received cholesterol for 110 days resulted in a marked regression of the lesions. Examination of his data reveals that in the six rabbits in which choline appeared to have a positive effect, the blood cholesterol levels were never very high and rapidly fell to the normal range, e.g.:

DAYS	0	20	60
	476 mg. %	149 mg. %	96 mg. %
	512	357	125

Where choline failed to influence the lesions, the blood cholesterol was high at the start and tended to stay high, e.g.:

DAYS	0	60
	1,265 mg. %	1,250 mg. %
	900	344

In the group taken off cholesterol and maintained on a regular diet without choline, the cholesterol values with one exception were over 300 mg. per cent at the end of sixty days, and the rate of fall was very slow. It would seem, therefore, that there is a strong correlation between the rate of fall of



the blood cholesterol levels and the efficacy of choline in affecting the regression of lesions. The failure of choline to act in four out of ten rabbits might well be accounted for on the basis of individual differences among rabbits, which are known to respond in varying fashion to cholesterol feeding. Steiner's results have been amply confirmed by Morrison and Rossi.<sup>16</sup>

By means of cholesterol tolerance tests<sup>17, 18</sup> we have been able to show that there is a marked difference in the response of different species to a standard load of their homologous cholesterol administered intravenously. We have found that in comparison with the chicken and the rat, the rabbit is much less capable of dealing with intravenously injected cholesterol and requires three to four times longer to dispose of an equivalent load of cholesterol. Kendall<sup>19</sup> and associates have published similar data using an artificially prepared emulsion of cholesterol. It appears therefore that the ability of any species to remove cholesterol from the arterial intima is related to its ability to metabolize cholesterol. Thus the chicken, which removes injected cholesterol from its blood stream about three times as fast as the rabbit, also shows a very rapid fall of blood cholesterol to normal after cessation of cholesterol feeding and a more rapid regression of lesions than does the rabbit. That this process may be accelerated by exogenous factors is well demonstrated by the effect of choline in the rabbit, and by under-nutrition in man. The existence of a physiologic mechanism for the removal of fat and cholesterol from the vascular wall is well supported by the evidence cited and is clearly suggested by the results of the present investigation. We are therefore compelled to abandon our "static" concepts concerning the nature of atherosclerosis and to apply instead the dynamic principles of modern metabolic theory concerning the behavior of the lipids in other parts of the body. Body fat is now known to be in a constant state of flux; it is laid down in depots and is constantly moved and replaced, converted or destroyed as required.<sup>20</sup> It would appear from the present study that under certain circumstances the lipid content of atheromatous plaques is subject to similar metabolic forces. Further studies on this aspect of the problem with more refined metabolic tools are the sine qua non of therapeutic researches in this field.

#### SUMMARY

1. Prolonged feeding of a diet containing 2 per cent cholesterol in cotton-seed oil for a period of twenty-four weeks resulted in progressive elevation of the blood cholesterol levels. There was increasing severity of the atherosclerosis for a period of fifteen weeks and then a levelling off, with a further increase in severity during the last two weeks of the experiment.

2. Cessation of cholesterol feeding after ten weeks is followed by a very rapid decline in the blood cholesterol levels to normal within three weeks. There was also a gradual regression in the severity of the lesions over a fourteen-week period. It appears that early lesions may be completely resorbed upon cessation of cholesterol feeding, while more severe lesions undergo regressive and reparative changes.

3. There appeared to be little difference in the rate of regression or disappearance of the aortic lesions in birds placed on a normal mash diet and those placed on a low fat, cholesterol-free diet following cessation of cholesterol feeding.

4. There is a wide spectrum of histologic changes resulting from prolonged cholesterol feeding, ranging from increase in the ground substance of the intima, with infiltration of sudanophile material, to very extensive foam cell plaques, hyaline and cartilaginous metaplasia, and heavy deposits of calcium in granules and plates.

5. Cessation of cholesterol feeding is followed by fibrotic changes in lesions of both the thoracic and abdominal portions of the aorta, by the disappearance and diminution of foam cells and fat, and by the calcification of atheromatous abscesses. We also noted the presence of scavenger-like, fat-filled cells in the intima.

6. The controls showed few gross lesions but numerous microscopic ones. Scattered focal deposits of sudanophile material were seen in the intima and inner portions of the media of the thoracic aorta. In the abdominal aorta the spontaneous lesions were characterized by fibrosis of the intima, with sudanophile deposits and calcium granules at the intimal-medial junction.

7. The presence of a physiologic mechanism concerned with the regression of atheroma in animals and man is discussed, and the application of modern dynamic principles of lipid metabolism to this mechanism is considered.

We are indebted to Miss C. Bolene (D. V. Dauber Memorial Research Assistant), Miss Lorraine Adams, Miss Eileen Arnold, Miss Marilyn Dudley, and Mr. William Foote for technical assistance in this study.

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# THE LACK OF EFFECT OF TWEEN 80 ON THE ABSORPTION OF ALUMINUM AND SODIUM PENICILLINS

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THE widespread use of oral penicillin despite the fact that only a fraction of an administered dose is absorbed from the gastrointestinal tract makes desirable the investigation of factors that may favor the absorption of penicillin from the gut. The present study reports the comparative plasma concentrations of penicillin that resulted from the oral administration of aluminum\* and sodium penicillins with and without orally administered Tween 80 (polyoxyethylene sorbitan monooleate).

The Tweens are a series of polyoxyalkylene derivatives of the hexitols, mannitol and sorbitol, and their anhydrides, partially esterified, that are classified as dispersing agents.<sup>1</sup> Such compounds might theoretically increase the absorption of substances from the gastrointestinal tract by lowering surface tension and increasing dispersion. It has been reported that one of these compounds, Tween 20, given by mouth and parenterally, prolonged the penicillin plasma concentrations following oral and parenterally administered penicillin.<sup>2</sup> Tween 80, differing only in the fact that oleic acid is esterified with the hexitan in place of lauric acid, has been fed to human patients in amounts as large as 15 Gm. per day without evidence of toxicity and furthermore has proved to be effective in increasing the absorption of fats and fat-soluble substances in certain pathologic conditions marked by impaired gastrointestinal absorption.<sup>3</sup> Because of its nontoxicity and its proved efficacy in human beings, Tween 80 was chosen for this study to determine whether it had any influence on the absorption of orally administered penicillin.

## MATERIALS AND METHODS

Twelve male subjects, varying in age from 33 to 78 years, afebrile and free of obvious renal, hepatic, or gastrointestinal dysfunction, were selected from the medical wards and divided into two groups of six each. One group was given aluminum penicillin with and without Tween 80 and the other was given sodium penicillin with and without Tween 80, each patient serving as his own control in determining the effect of the dispersing agent on penicillin plasma concentrations.

Penicillin was administered orally in a dose of 200,000 units every three hours for nine doses, and blood samples were drawn into heparin-wetted syringes at one-half, one, two, and three hours after the ninth dose. No medication was given for twenty-four hours and then 200,000 units of penicillin and 2 Gm. of Tween 80 were given together every three hours for nine doses. After the ninth dose of the combination of drugs, blood samples again were

From the Philadelphia General Hospital.

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drawn at one-half, one, two, and three hours. The ninth dose of medication in each phase of the study was given at the same time of day and in the same relationship to the ingestion of food, so that the penicillin time-dose response curves in the two phases of the study can be compared to determine the effect of Tween 80. The same lots of aluminum and sodium penicillins were employed throughout the study. Penicillin assays were done by the Kirby-Rantz modification of the Rammelkamp serial dilution method,<sup>4</sup> employing as the test organism *Streptococcus* 98.

### RESULTS

In Table I are presented the data obtained following the use of sodium penicillin. It is apparent that in three patients (E. U., B. L., and P. L.) the penicillin plasma concentrations were actually lower when Tween 80 was administered; in two patients (G. J. and J. M.) the concentrations were higher; and in one patient (J. M.) the concentrations were equal to those obtained when penicillin was given alone. The average values from the six patients show the concentrations after penicillin alone to be slightly higher than those after penicillin and Tween 80.

TABLE I. SODIUM PENICILLIN: 200,000 UNITS\* ORALLY; COMPARISON OF PENICILLIN PLASMA CONCENTRATIONS WITH AND WITHOUT TWEEN 80

PATIENT	AGE	TWEEN 80†	PENICILLIN PLASMA CONCENTRATIONS (HOURS AFTER ADMINISTRATION)			
			½	1	2	3
G. J.	67	Without	0.19	0.19	0.38	0.25
		With	0.76	0.76	0.76	0.76
J. M.	78	Without	1.00	1.50	1.50	1.50
		With	2.00	1.00	1.50	1.00
J. M.	33	Without	0.25	0.19	0.25	0.13
		With	0.76	0.50	0.19	0.13
E. U.	72	Without	1.50	1.50	1.00	1.00
		With	---	0.38	0.38	0.25
B. L.	36	Without	0.97	1.65	1.25	0.95
		With	0.47	0.80	0.37	0.30
P. L.	45	Without	0.55	1.20	0.32	0.19
		With	0.30	0.55	0.25	0.17
Average		Without	0.74	1.03	0.78	0.67
		With	0.85	0.66	0.57	0.43

\*Two 100,000 unit tablets each buffered with 0.35 Gm. calcium carbonate.

†16 Gm. per day administered as four 0.5 Gm. gelatin capsules every three hours.

Table II shows similar data after the use of aluminum penicillin. In two patients (H. M. and C. B.) the plasma concentrations of penicillin are lower after the use of Tween 80; in two patients (J. W. and R. M.), slightly higher; and in two patients (S. B. and E. H.), approximately equal to those observed after the use of aluminum penicillin alone. The average values from the six patients are practically identical.

### DISCUSSION

The Tweens are painful and irritating when injected intramuscularly, so even if effective in elevating penicillin plasma concentrations as claimed for Tween 20,<sup>2</sup> this route of administration would be impractical. Furthermore, the amount of Tween 80 absorbed from the gut and excreted by the kidneys is

TABLE II. ALUMINUM PENICILLIN: 200,000 UNITS\* ORALLY; COMPARISON OF PENICILLIN PLASMA CONCENTRATIONS WITH AND WITHOUT TWEEN 80

PATIENT	AGE	TWEEN 80†	PENICILLIN PLASMA CONCENTRATIONS (HOURS AFTER ADMINISTRATION)			
			½	1	2	3
J. W.	68	Without	1.60	1.60	1.60	1.60
		With	1.60	3.02	2.50	2.50
R. M.	67	Without	0.25	0.50	0.25	0.25
		With	0.31	0.76	0.76	0.25
S. B.	50	Without	0.19	0.19	0.25	0.25
		With	0.25	0.38	0.19	0.13
H. M.	59	Without	1.00	2.00	0.76	0.38
		With	0.76	0.76	0.50	0.76
C. B.	78	Without	3.00	3.00	3.00	1.50
		With	1.50	0.76	2.00	1.50
E. H.	71	Without	0.45	0.78	0.70	0.80
		With	0.47	0.98	0.75	1.20
Average		Without	1.08	1.34	1.09	0.79
		With	0.81	1.11	1.11	1.05

\*Four 50,000 unit tablets, each buffered with 0.3 Gm. sodium benzoate.  
†16 Gm. per day administered as four 0.5 Gm. gelatin capsules every three hours.

so small<sup>5</sup> that a carinamide-like effect ascribed to Tween 20<sup>2</sup> seems unlikely. The sole anticipation of an effect of Tween 80 on the absorption of penicillin was predicated on its activity as a dispersing agent within the gut. The observations made in twelve patients under the conditions outlined indicate that Tween 80 exerted no effect on the absorption of penicillin from the gastrointestinal tract.

Aluminum and sodium penicillin were not compared in the same patients, but it is apparent that the penicillin plasma concentrations observed in similar groups of patients were approximately equal during the three-hour period studied. A subsequent investigation will be directed toward comparing the maintenance of penicillin plasma concentrations for periods longer than three hours. It should be noted that the penicillin plasma concentrations were unexpectedly high; except initially, they exceeded those resulting from the intramuscular injection of 50,000 units of penicillin (crystalline penicillin in aqueous solution)<sup>6</sup> The concentrations observed may be explained in part by the ages of the patients studied or by the possibility of penicillin accumulation in the circulation during the twenty-four hours of medication prior to the time-dose response values here recorded. Unfortunately there is no body of data in the literature with which to compare the results of this study, but it would be unwarranted to assume that penicillin plasma concentrations of the magnitude herein reported obtain in all patients to whom 200,000 units of penicillin are orally administered every three hours.

#### CONCLUSIONS

Tween 80 (polyoxyethylene sorbitan monooleate) in oral doses of 16 Gm. per day did not enhance the gastrointestinal absorption of penicillin as determined by the penicillin plasma concentrations resulting from the oral administration of the antibiotic with and without Tween 80.

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## LABORATORY METHODS

### EVALUATION OF A MODIFIED SUMNER'S METHOD (DINITROSALICYLIC ACID) FOR DETERMINATION OF GLUCOSE IN URINE

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SUMNER<sup>1, 2, 3</sup> has described methods for the determination of glucose in urine and blood, using dinitrosalicylic acid as an oxidizing agent. The reduced dinitrosalicylic acid is determined colorimetrically. This method has been employed extensively by Exton<sup>4, 5</sup> for the determination of both urinary glucose and, by modification, other sugars found in urine. Recently, Leech and Woodford<sup>6</sup> have utilized this principle in a simple and rapid method for the approximate estimation of blood glucose.

The present paper deals with the employment and detailed evaluation of this method, slightly modified, for the determination of glucose in urines that do not contain other reducing sugars.

#### PROCEDURE

The following procedure was found to be satisfactory.

*Preparation of the Reagent.*—Solution 1: 120 Gm. of sodium potassium tartrate and 6 Gm. of phenol (crystalline) are dissolved in 350 c.c. of water. A solution of 6 Gm. of sodium bisulfite in 60 c.c. of water is added.

Suspension 2. 20 Gm. of 3-5 dinitrosalicylic acid monosodium salt are suspended in 800 c.c. of water, care being taken to avoid large lumps.

Solution 3: 40 Gm. of sodium hydroxide are dissolved in 400 c.c. of water and cooled.

Forty cubic centimeters of Solution 3 are added to Suspension 2 and the mixture is shaken until the salt has dissolved. This is completed in a very short time if there are no large lumps in the suspension. Now Solution 1 is added and mixed well with the solution of dinitrosalicylate. Finally 320 c.c. of Solution 3 are added in three or four portions. The mixture is shaken after the addition of each of these portions. If all of the sodium hydroxide is added at once, a precipitate may be formed which is very difficult to redissolve. The volume is made up to 2,000 c.c. with water.

This reagent is kept for two weeks at 25 to 30° C. before use.

*The Reduction and the Colorimetric Determination.*—Ten cubic centimeters of reagent are pipetted into a 19 mm. test tube and 0.1 c.c. of urine is added from a blood capillary pipette. The pipette is rinsed in the reagent and the mixture is stirred with the pipette or a glass rod. To another 10 c.c. of reagent in a similar test tube is added 0.1 c.c. of water. The tubes are heated in boiling water for three minutes and cooled to room temperature.

The optical density is determined by spectrophotometry\* in a 19 mm. tube, using the reagent blank as a standard. For glucose concentrations below 0.6

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\*Different types of colorimeters may be used. The described procedure is based upon the employment of a Coleman Junior Spectrophotometer which proved very satisfactory for this purpose. It may be operated rapidly and gives a reasonable degree of accuracy.



per cent the wave length  $540\text{ m}\mu$  is used; for concentrations above this value,  $700\text{ m}\mu$  is used. The glucose concentration is found from a standard curve or from Table III. Finally, the temperature of the solutions is measured and, if necessary, a correction made by means of Table IV.

#### RESULTS AND COMMENT

It was found that the results vary, depending on the method of preparing the reagent. The amount of color developed is further dependent upon the age of the batch of reagent used. From Fig. 1 it will be seen that the intensity of

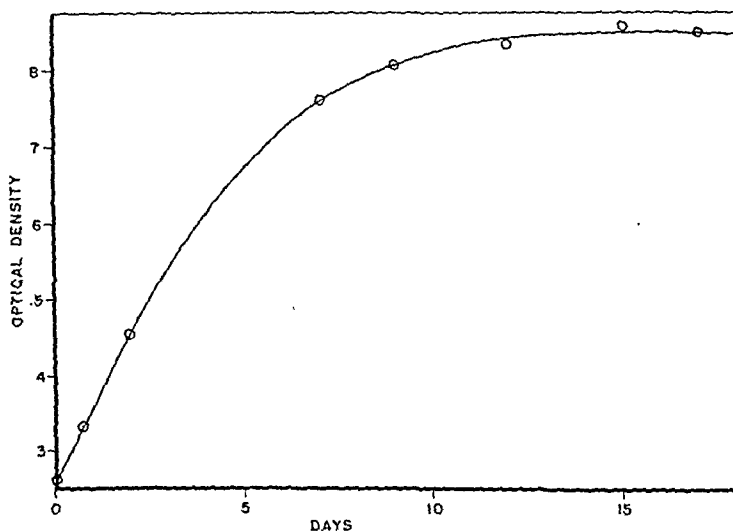


Fig. 1.—The density of color obtained after boiling, using the same batch of reagent at different periods of time after preparation. Constant glucose concentration.

the color, when the reagent is kept at  $27^{\circ}\text{C}$ ., increases for the first twelve days or so and thereafter remains constant. It is thus necessary to leave the freshly prepared reagent for about two weeks at this temperature before use. At lower temperatures a longer time is required, while at  $37^{\circ}\text{C}$ . the aging probably will be complete in one week. We have tried to age the reagent in five hours in boiling water. This procedure, although suitable for emergency use, is not recommended, since it results in a rather dark-colored reagent which is unsuitable for exact work. As shown in Table I, the reagent, once it has been aged, is stable for at least three months at room temperature when kept in a brown bottle in the dark.

TABLE I. DENSITIES FOUND, USING DIFFERENT BATCHES OF REAGENT  
All Glucose Concentrations Are 0.5%. Each Value Is the Average of 5 Determinations

DATE OF PREPARATION OF REAGENT	AGE OF REAGENT (DAYS)	DENSITY AT $22^{\circ}\text{C}$ .
3/6	34	0.800
3/6	77	0.826
3/6	99	0.794
4/16	15	0.840
4/16	37	0.820
4/16	58	0.798
5/24	13	0.817
5/24	20	0.804

For pipetting the reagent, an automatic 10 c.c. pipette\* is convenient.

In these experiments 0.1 c.c. of urine† was used. The pipetting of this volume with a 0.1 c.c. capillary blood pipette is the largest single source of error in the method. The pipettes used in this work (Sargent, Chicago), as received from the manufacturer, are said to measure 0.1 c.c.  $\pm$  0.005 cubic centimeter. All of thirty pipettes tried were found to be within these limits.

When working with pure glucose solutions or with urines containing much glucose and only small amounts of other reducing substances, the mixture of urine and reagent may, if desired, be left at room temperature for an hour before boiling. Table II shows the values for optical density where different periods of time before boiling were employed.‡ In the case of very concentrated solutions (10 per cent glucose) a certain amount of the brown color develops during the first hour at room temperature. This does not affect the final result.

TABLE II. VALUES FOR OPTICAL DENSITY FOUND WHEN A MIXTURE OF A PURE GLUCOSE SOLUTION AND THE REAGENT REMAINED AT ROOM TEMPERATURE FOR DIFFERENT PERIODS OF TIME BEFORE BOILING

TIME (MIN.)	DENSITY
0	0.630
5	0.642
10	0.652
30	0.653
60	0.642

If large amounts of creatinine are present, reduction takes place at room temperature within a short time, as indicated in Fig. 2. The amount of color developed by physiologic concentrations of creatinine is rather small and usually can be neglected. If one, however, is interested in determining concentrations of glucose in the range of 0.1 to 0.4 per cent, the reduction by creatinine must be taken into consideration and the tubes should be put into the boiling water as soon as possible after mixing urine and reagent.

A boiling time of three minutes was found satisfactory. The variation of the color obtained by varying the boiling time from 2.5 to 4 minutes is seen in Fig. 3. One should make sure that there is proper contact between test tubes and boiling water. If many tubes are placed in the water bath without sufficient space between them, the tubes in the middle will remain at a lower temperature than the others and erroneous results will follow.

After boiling, the tubes are placed in cold water. It is convenient to have two vessels for this purpose. The tubes are first placed for a few minutes in one with running tap water and thereafter in the other containing water at room temperature. In this way rapid cooling is obtained and all the tubes will be at room temperature for the colorimetric determination. As will be shown later, this is essential, since the intensity of the color depends upon temperature.

\*If mouth pipettes are used it must be remembered that the reagent is caustic and poisonous.

†It is usually unnecessary to filter the urine. Even urines containing large amounts of precipitates will usually give clear solutions on boiling in the strongly alkaline reagent.

‡The use of 19 mm test tubes for heating the mixture of reagent and urine in boiling water obviates any appreciable loss by evaporation.

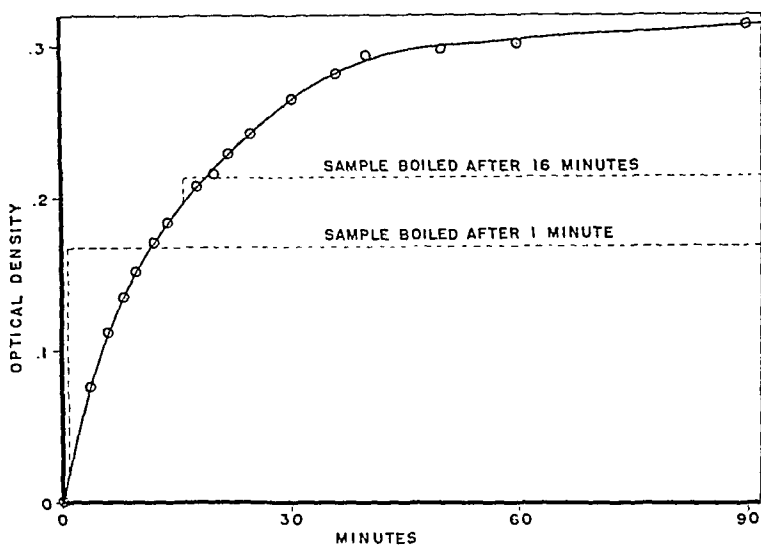


Fig. 2.—Development of color expressed as density at 540  $m\mu$  by a solution of creatinine corresponding to 3.8 per cent in 0.1 c.c. of urine added to the reagent and standing at 25° C. Two samples were taken out and boiled and thereby the development of color was stopped at a lower density.

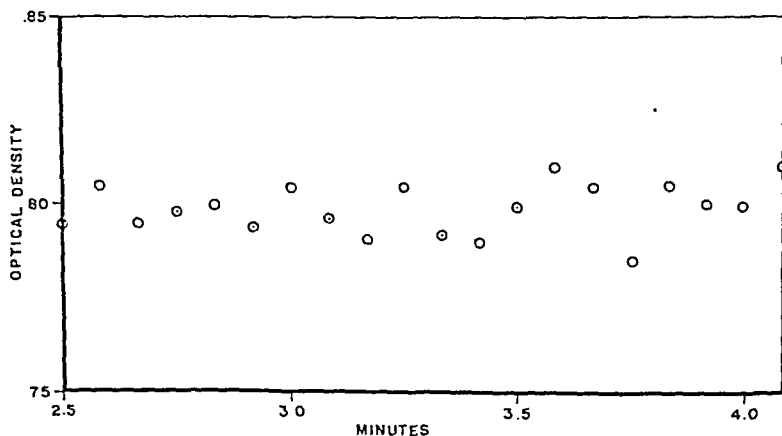


Fig. 3.—Twenty test tubes containing samples of the same mixture of glucose and reagent were boiled for different periods of time. The variation in density, determined at 540  $m\mu$  after cooling, reflects the statistical uncertainty of the determination. In addition, there is possibly a slight increase in color from 2.5 minutes to 4.0 minutes.

For accurate work, the colorimetric determination should be carried out within one or two hours after boiling, since there is a small increase in the intensity of the color during the first twenty-four hours as shown in Fig. 4. After twenty-four hours there is a decrease in color. The initial value is obtained again in a couple of days.

The method has the advantage that the entire range of glucose concentrations usually encountered (0.2 to 10 per cent) can be determined without the necessity of making different dilutions of urine. This not only saves time but

also eliminates an important source of mistakes in computation. Using the Coleman Junior Spectrophotometer and 19 mm. test tubes, it was found that the whole range could be covered by employing 0.1 c.c. of urine to 10 c.c. of the reagent and determining the developed colors at two different wave lengths.

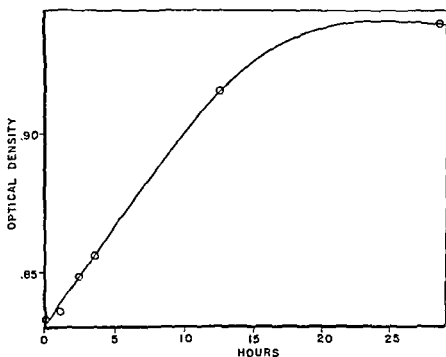


Fig. 4.—The density after boiling as a function of the time during which the tubes remained at room temperature. Each point is the average of five determinations.

For lower concentrations, up to 0.6 per cent glucose, 540  $m\mu$  was found to be optimal, whereas for concentrations in the range 0.6 to 10 per cent, 700  $m\mu$  was found to be optimal. For other types of colorimeter, or for this colorimeter with a different-sized tube, a different wave length may be found to be better. To avoid mistakes it is preferable always to record the densities of all samples at both of the two wave lengths.

It is ordinarily recommended that wave lengths be employed for which the substance to be determined has a maximum or minimum of absorption. This could not be done in this instance since no such maximum or minimum is present.

Using other types of colorimeters, it may be necessary to dilute the reaction mixture. If so, dilution with water should be avoided, since the color is unstable except in strong solutions of sodium hydroxide. Dilution may be made with 5 per cent sodium hydroxide which does not influence the stability of the color. In this case the reagent blank, used as a standard, must be diluted in the same proportion. It is easier, however, to dilute with the reagent itself, in which case the reagent blank can be used unchanged.

The colors of the solutions were found to be dependent to a considerable degree upon temperature. Thus the density of the reagent increased 20 per cent with an increase in temperature from 20° to 30° C., whereas the density of the strongly colored solutions resulting from high sugar concentrations increases only 9 per cent with the same change in temperature. Therefore it is necessary, first, to keep the reagent blank and the reaction mixture at the same temperature and, second, to take this temperature into consideration in the calculation. To be safe, it is always best to use one reagent blank with

TABLE III. THE GLUCOSE CONCENTRATION AS A FUNCTION OF THE OPTICAL DENSITY  
 Along With the Probable Value of the Concentration Are Given Upper and  
 Lower Limits, Calculated in Such a Way That the Probability Is About  
 99 Per Cent That the True Value Is to Be Found Within Them

TEMPERATURE: 22° C.							
% GLUCOSE OR EQUIVALENT AMT. OF OTHER REDUCING SUBSTANCE				% GLUCOSE OR EQUIVALENT AMT. OF OTHER REDUCING SUBSTANCE			
DENSITY	PROBABLE VALUE	LOWER LIMIT	UPPER LIMIT	DENSITY	PROBABLE VALUE	LOWER LIMIT	UPPER LIMIT
540 M $\mu$							
.01	.11	.00	.20	.52	.36	.33	.39
.02	.12	.00	.21	.54	.36	.33	.39
.03	.13	.05	.21	.56	.37	.34	.40
.04	.13	.07	.21	.58	.38	.35	.41
.05	.14	.08	.21	.60	.39	.36	.42
.06	.14	.09	.22	.62	.40	.37	.43
.07	.15	.10	.22	.64	.41	.38	.44
.08	.16	.10	.22	.66	.42	.39	.45
.09	.16	.11	.22	.68	.42	.39	.45
.10	.17	.12	.23	.70	.43	.40	.46
.12	.18	.13	.23	.72	.44	.41	.47
.14	.19	.15	.24	.74	.45	.42	.48
.16	.20	.16	.24	.76	.46	.43	.50
.18	.21	.17	.25	.78	.47	.44	.51
.20	.21	.18	.25	.80	.48	.45	.52
.22	.22	.19	.26	.82	.49	.45	.53
.24	.23	.20	.26	.84	.50	.46	.54
.26	.24	.21	.27	.86	.51	.47	.55
.28	.25	.22	.28	.88	.53	.49	.57
.30	.26	.23	.29	.90	.54	.50	.58
.32	.27	.24	.30	.92	.55	.51	.59
.34	.28	.25	.31	.94	.57	.53	.61
.36	.29	.26	.32	.96	.58	.54	.62
.38	.30	.27	.33	.98	.59	.55	.63
.40	.30	.27	.33	1.00	.60	.56	.64
.42	.31	.28	.34				
.44	.32	.29	.35				
.46	.33	.30	.36				
.48	.34	.31	.37				
.50	.35	.32	.38				
700 M $\mu$							
.06	.50	.40	.60	.26	2.2	2.0	2.4
.07	.60	.50	.70	.27	2.3	2.1	2.5
.08	.65	.55	.75	.28	2.4	2.2	2.6
.09	.75	.65	.85	.29	2.5	2.3	2.7
.10	.80	.70	.90	.30	2.6	2.4	2.8
.11	.90	.80	1.00	.32	2.8	2.6	3.0
.12	.95	.85	1.05	.34	3.1	2.8	3.4
.13	1.05	.95	1.15	.36	3.3	3.0	3.6
.14	1.15	1.00	1.30	.38	3.5	3.2	3.8
.15	1.20	1.05	1.35	.40	3.7	3.4	4.0
.16	1.30	1.15	1.45	.42	3.9	3.6	4.2
.17	1.40	1.25	1.55	.44	4.2	3.8	4.6
.18	1.50	1.35	1.65	.46	4.4	4.0	4.8
.19	1.60	1.45	1.75	.48	4.7	4.3	5.1
.20	1.65	1.50	1.80	.50	4.9	4.5	5.3
.21	1.70	1.55	1.85	.52	5.2	4.8	5.6
.22	1.80	1.65	1.95	.54	5.4	5.0	5.8
.23	1.90	1.75	2.05	.56	5.7	5.2	6.2
.24	2.00	1.85	2.15	.58	5.9	5.4	6.4
.25	2.10	1.90	2.30	.60	6.2	5.7	6.7

TABLE III—CONT'D

TEMPERATURE: 22° C.							
% GLUCOSE OR EQUIVALENT AMT. OF OTHER REDUCING SUBSTANCE				% GLUCOSE OR EQUIVALENT AMT. OF OTHER REDUCING SUBSTANCE			
DENSITY	PROBABLE VALUE	LOWER LIMIT	UPPER LIMIT	DENSITY	PROBABLE VALUE	LOWER LIMIT	UPPER LIMIT
.62	6.4	5.9	6.9	.82	9.2	8.5	9.9
.64	6.7	6.2	7.2	.84	9.5	8.7	10.3
.66	6.9	6.4	7.4	.86	9.9	9.2	10.6
.68	7.2	6.7	7.7				
.70	7.4	6.9	7.9				
.72	7.7	7.2	8.2				
.74	8.0	7.4	8.6				
.76	8.2	7.7	8.9				
.78	8.6	8.0	9.2				
.80	8.9	8.3	9.5				

each lot of tubes boiled. In this way one can be sure that there is no great temperature difference between reagent blank and reaction mixture. Table III is calculated for a temperature of 22° C. For other temperatures a correction\* must be made. Table IV shows the values for this correction. These are applicable, no matter what type of colorimeter is used.

TABLE IV. TEMPERATURE CORRECTIONS

GLUCOSE CONCENTRATION FOUND FROM TABLE III (%)	TEMPERATURE (C.)				
	15°	20°	25°	30°	35°
	CORRECTION (% GLUCOSE)				
.1	.00	.00	.00	.00	.01
.2	.01	.00	.00	.01	.02
.3	.02	.00	.00	.02	.03
.4	.02	.01	.01	.02	.04
.5	.03	.01	.01	.03	.05
.6	.03	.01	.01	.03	.06
.8	.04	.01	.01	.04	.08
1.0	.05	.02	.02	.05	.10
1.5	.08	.02	.03	.08	.15
2.0	.10	.03	.04	.10	.20
2.5	.13	.04	.06	.13	.25
3.0	.15	.05	.08	.15	.30
4.0	.20	.06	.09	.20	.40
5.0	.25	.08	.12	.25	.50
6.0	.3	.1	.1	.3	.6
7.0	.4	.1	.1	.4	.7
8.0	.4	.1	.2	.4	.8
9.0	.5	.1	.2	.5	.9
10.0	.5	.2	.2	.5	1.0

For computation of the sugar concentration from the density, a set of two curves can be used, one for each of the two wave lengths. For routine use, however, a table such as Table III is more convenient and is less subject to mistakes in application.

It should be pointed out that the relation between density and glucose concentration is not linear. It is especially remarkable that small amounts of glucose give no color at all. Thus a density reading of approximately zero means

\*For clinical work in which approximate values are satisfactory, and in which laboratory temperatures vary only a few degrees, this correction is unnecessary.

not that there is no glucose in the urine but that there is less than about 0.1 per cent. For clinical purposes glucose concentrations as low as this are usually of no significance.

For the purpose of rough estimations, where an error of  $\pm 20$  per cent is unimportant, the standard curve may be determined by applying the previously described procedure to known solutions of glucose. If limits of error of about  $\pm 10$  per cent are required, great care must be taken to make the standard curve exact enough. In this work the following procedure was employed.

The glucose content of a commercial dextrose preparation was determined by polarimetry. From this preparation a solution was made up, containing a known amount of glucose per gram. A portion of this solution was weighed out, water added to make a total volume of 1 c.c., and 100 c.c. of reagent were added. After thorough mixing, the solution was divided into ten portions in each of ten test tubes and heated in boiling water for three minutes. After cooling, the color was determined in the usual manner using ten different tubes of reagent blank. The average of the ten readings makes up one point of the curve. The figures in Table III are read from this standard curve.

A number of calibrated colorimeter tubes were used for determination of the density of the same solution. They were found to vary slightly, the standard deviation being  $\pm 0.5$  per cent. Other colorimeter errors were found to cause a standard deviation of  $\pm 1$  per cent. The errors in determining the points on the standard curve, made in the previously described manner, were found to cause a standard deviation in the apparent glucose concentration of  $\pm 0.4$  per cent for values higher than 0.3 per cent glucose.

The upper and lower limits of error, given in Table III, were calculated from these figures together with the previously mentioned pipette errors. These limits are determined in such a way that the probability is about 99 per cent that the true value is to be found within them.

The method described is sufficiently accurate for clinical work. The Benedict titration, in the form in which it is carried out in many clinical laboratories, that is, without any precautions to prevent the oxidizing influence of the air, is much less accurate. Titrations of eleven portions of a single sample of urine from a diabetic patient showed the following percentages of glucose: 1.3, 1.4, 0.9, 1.3, 1.2, 0.8, 1.0, 1.1, 1.9, 1.9. The proportion between the highest and the lowest of these values is more than 2:1.

*Interference by Substances Other Than Glucose.*—In the present work, it was assumed that no sugars other than glucose were present. The fact that different sugars give different reduction rates with the dinitrosalicylic reagent has been utilized by Exton<sup>6</sup> for the identification of these substances in urines. We have found, however, that the relative rates of reduction depend not alone upon which sugar is present but also to some extent upon the concentration. Thus, further investigations must be carried out before the method can be adapted to routine use for the differentiation of melliturias.

Other reducing substances present in urine, such as creatinine, uric acid, phenols, and amino acids, may interfere with the determination of glucose by

most of the methods in common use. In order to test their effect on the present method, the following substances were added to the reagent in amounts corresponding to very high concentrations in the urine: 11 per cent phenol, 22 per cent salicylic acid, 10 per cent acetone, 30 per cent urea, 10 per cent oxalic acid, and 3 per cent uric acid. None of these substances gave any measurable color. The color produced by creatinine has been mentioned previously; 0.16 per cent creatinine in the urine was found to give a density of 0.03 after standing at room temperature for an hour. For most clinical purposes reduction by creatinine can be neglected.

A number of urines from dogs were determined both by this method and by the Benedict titration (Table V). In about half of the "normal" urines the Benedict method yielded considerable amounts of reducing substances, the maximum being 0.9 per cent expressed as glucose equivalent and values of about 0.6 per cent being very common. In one such urine no fermentation occurred with yeast; whereas a nonreducing urine to which was added 0.6 per cent glucose gave a slight fermentation. In contrast, by the dinitrosalicylic acid method much lower reducing values were found in the same urines, usually about half as much as by the Benedict titration, indicating a higher degree of specificity of the present method.

TABLE V. REDUCING POWER OF DIFFERENT SAMPLES OF DOG URINES AS DETERMINED BY THE BENEDICT METHOD AND BY THE DINITROSALICYLIC ACID METHOD  
Values Are Expressed as Per Cent Glucose Equivalent

BENEDICT	DINITROSALICYLIC ACID
0.1	Less than 0.1
0.25	0.1
0.7	0.26
0.33	0.15
0.25	0.1
0.6	0.27
0.6	0.24
0.83	0.44
0.6	0.33
Less than 0.1	Less than 0.1
0.4	0.14
0.36	0.12
0.58	0.36
0.56	0.30
0.40	0.36
0.51	0.23

#### SUMMARY

A modification of Sumner's method has proved very satisfactory as a routine method for the determination of glucose in urine. The procedure has the advantage that the entire range of glucose concentrations from 0.2 to 10 per cent is covered without using different dilutions of the urine. The limits of error are within  $\pm 10$  per cent for concentrations higher than 0.3 per cent glucose. For such concentrations the influence of nonsugar reducing substances can be largely neglected. This method is thus more specific than other routine methods. If sugars other than glucose are present, however, erroneous results will be obtained.



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# A SIMPLE METHOD FOR DETERMINING SULFONAMIDE SENSITIVITY IN VITRO AND ITS CLINICAL APPLICATION

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THIS communication describes a simple method for the determination of the relative sensitivity of a given bacterial strain to different sulfonamides. A method for this purpose has not heretofore come into clinical use, in part because various substances contained in the usual culture media, particularly peptone,<sup>1</sup> prevent a determination of the absolute antibacterial effect of sulfonamides. Special media which do not contain such inhibitors have been prepared,<sup>2-7</sup> but the techniques involved are too laborious for practical clinical application. Peptone-containing media however, if properly prepared, can be used for determining the relative antibacterial potency of various sulfonamides against a given bacterial strain. The method described below enables one not only to determine the sensitivity of a given bacterial strain to a series of sulfonamides but also to choose the most potent one.

## METHOD

Nutrient broth (Difco) containing 0.5 per cent peptone was adjusted to a pH of 8.2 in order to obtain maximum solubility of the sulfonamides. The amount prepared was sufficient to serve for all procedures of the entire study. For growth of the more fastidious bacteria the pH was lowered (pH 7.6) and horse serum (5 per cent) and glucose (1 per cent) were added. Serial dilutions of each of a 1,000 mg. per cent stock solution of the various sulfonamides were prepared in concentrations of 750, 500, 200, 100, 50, 25, and 10 mg. per cent respectively. One cubic centimeter of each of the eight dilutions was placed in Wassermann tubes. To each tube was added 0.1 c.c. of a bacterial suspension prepared by diluting an eighteen to twenty-four hour culture so that 0.1 c.c. contained 1,000 to 20,000 bacteria. A control tube containing broth and no sulfonamide was inoculated with 0.1 c.c. of the same bacterial suspension.

Such small inocula were used because for some strains large inocula are said to depress the activity of the sulfonamides.<sup>8</sup> The broth prepared as described was used to prepare all stock solutions, all the dilutions of the sulfonamides, and the bacterial cultures and dilutions. The tubes were incubated for twenty-four hours at 37° C. The lowest concentration of each sulfonamide which completely inhibited macroscopically visible growth was taken to be the bactericidal titer. The concentration of each sulfonamide which produced a recognizably lesser degree of turbidity than the control tube was considered to be the bacteriostatic titer.

We are aware of the fact that this method of reading an *in vitro* test is somewhat inaccurate. The last clear tube may contain a small number of viable bacteria, which may be destroyed within another twenty-four hours or may start to multiply after twenty-four hours have elapsed. Also, a tube macroscopically just as cloudy as the control tube might well contain fewer organisms than the latter. However, for practical purposes, these possibilities can be neglected, as will be shown in the discussion of the results.

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## RESULTS

Two hundred bacterial strains were studied. Markedly different action was observed by various sulfonamides on many strains of *Escherichia coli*, *Aerobacter aerogenes*, *Klebsiella pneumoniae*, *Eberthella typhi*, *Salmonella schottmülleri*, *Salmonella enteritidis*, *Bacillus proteus vulgaris*, and *Pseudomonas aeruginosa* and among some strains of *Staphylococcus aureus hemolyticus*, but only rarely on various strains of hemolytic streptococci and pneumococci. Some bacterial strains of each of these species were equally resistant while others were equally sensitive to all sulfonamides studied. Gram-negative cocci and gram-positive bacilli were not examined. Table I provides a few significant examples.

## CLINICAL OBSERVATIONS

The clinical importance of such tests for selecting the most effective sulfonamide is emphasized by the following representative case histories.

CASE 1.—A 25-year-old man with pneumonia of the right upper lobe was treated with large doses of sulfadiazine for three days without effect. At this time, the process extended to the right middle lobe. A pneumococcus Type 1, isolated from blood and sputum, was completely resistant in vitro to penicillin, sulfadiazine, sulfamerazine, and sulfamethazine. The bactericidal titer with sulfathiazole was 100 mg. per cent. Administration of sulfathiazole in adequate dosage led to recovery within thirty-six hours.

CASE 2.—A sequestrectomy was performed in a girl of 10 with osteomyelitis of the right tibia of four to five weeks' duration. Penicillin and sulfadiazine were administered without effect. Temperature remained high. A hemolytic, coagulase-positive *Staph. aureus*, isolated from the draining sinus in pure culture, was completely resistant in vitro to penicillin, sulfadiazine, sulfamerazine, and sulfamethazine. The bactericidal titer with sulfathiazole was 250 mg. per cent. Following sulfathiazole therapy, fever rapidly declined, drainage subsided, and the patient recovered.

CASE 3.—A 70-year-old woman with acute postoperative cystitis due to *Esch. coli* was treated for five days with full doses of sulfadiazine without effect. The strain was completely resistant in vitro to sulfadiazine, sulfathiazole, sulfamethazine, and streptomycin. The bactericidal titer with Nu 445 (3,4-dimethyl-5-sulfanilamido-isoxazole) was 100 mg. per cent. There was complete clinical and bacteriologic cure after three days of treatment with Nu 445.

CASE 4.—A 40-year-old woman with acute pyelonephritis and cystitis due to *Esch. coli* was treated for six days with full doses of sulfadiazine without improvement. The bactericidal titer with sulfadiazine, sulfathiazole, and sulfamethazine was 500 mg. per cent, but with Nu 445 it was 250 mg. per cent. Clinical and bacteriologic cure was achieved within six days with Nu 445.

CASE 5.—Following prostatectomy a 50-year-old man developed acute cystitis and bilateral pyelonephritis due to *A. aerogenes* and *Ps. aeruginosa*. Prolonged therapy with sulfadiazine and later with sulfamerazine was ineffective. Both strains were resistant in vitro to sulfadiazine, sulfamerazine, and sulfathiazole. The bactericidal titer of the *A. aerogenes* with sulfamethazine was 100 mg. per cent, of the *Ps. aeruginosa*, 250 mg. per cent. Treatment with this drug rapidly cured the infection.

CASE 6.—A 66-year-old woman following perineorrhaphy developed acute cystitis due to *Ps. aeruginosa* and *B. faecalis alcaligenes*. Penicillin in large doses was without effect. In vitro tests showed a bactericidal titer for the *B. alcaligenes* of 100 mg. per cent with sulfadiazine, sulfathiazole, and sulfamethazine. The *Ps. aeruginosa* was completely resistant to

TABLE I. COMPARATIVE IN VITRO SENSITIVITY OF SEVERAL STRAINS OF A GIVEN SPECIES OF BACTERIA TO VARIOUS SULFONAMIDES (SIZE OF INOCULUM, 2,000 TO 8,000 BACTERIA; TITERS IN MG % OF DRUG IN NUTRIENT BROTH)

STRAIN	SD			SM			SMT			ST			PST			Nu 445		
	c	st	st	c	st	st	c	st	st	c	st	st	c	st	st	c	st	st
Esch. coli 1	250	50		250	50		25	25		500	100		750	250		750	500	
Esch. coli 2	250	100		250	100		250	100		100	50		500	250		1,000	500	
Esch. coli 3	1,000	250		750	250		750	250		500	250		750	500		100	25	
Esch. coli 4	1,000	750		1,000	1,000		500	100		50	25		500	250		>1,000	1,000	
A. aerogenes 1	>1,000	1,000		>1,000	1,000		100	100		500	250		500	250		>1,000	>1,000	
A. aerogenes 2	>1,000	>1,000		>1,000	>1,000		750	500		250	250		750	500		>1,000	>1,000	
A. aerogenes 3	750	750		750	750		500	250		100	50		500	500		>1,000	>1,000	
A. aerogenes 4	1,000	750		>1,000	>1,000		250	100		500	250		750	750		>1,000	>1,000	
B. proteus 1	250	25		500	500		100	50		750	50		750	250		100	25	
B. proteus 2	750	750		1,000	750		500	100		500	250		500	250		100	25	
B. proteus 3	>1,000	>1,000		>1,000	>1,000		500	250		100	25		250	100		750	500	
B. proteus 4	250	250		500	250		500	250		750	250		750	250		1,000	750	
Ps. aeruginosa 1	>1,000	>1,000		>1,000	>1,000		500	250		>1,000	>1,000		>1,000	>1,000		>1,000	>1,000	
Ps. aeruginosa 2	>1,000	1,000		>1,000	>1,000		1,000	750		250	100		500	250		>1,000	>1,000	
Ps. aeruginosa 3	1,000	1,000		1,000	750		750	500		500	250		750	750		>1,000	>1,000	
Ps. aeruginosa 4	1,000	1,000		>1,000	>1,000		1,000	500		750	750		750	750		100	50	
Staph. aureus 1	500	250		250	100		500	500		1,000	750		>1,000	1,000		1,000	1,000	
Staph. aureus 2	1,000	750		>1,000	1,000		100	50		250	250		750	750		1,000	1,000	
Staph. aureus 3	100	100		250	250		500	500		750	500		750	750		>1,000	>1,000	
Staph. aureus 4	750	500		750	500		500	250		100	25		500	100		>1,000	>1,000	

SD, Sulfadiazine; SM, Sulfamethazine; SMT, Sulfamethazine; ST, Sulfathiazole; PST, Sulfathiazole; Nu 445, 3,4-dimethyl-5-sulfamidoisoxazole.

c, Bacterioid titer, st, Bacteriostatic titer.

All strains were freshly isolated from human infections. Only those showing marked difference in sensitivity against various sulfonamides are listed.

Titers of 250 mg. per cent or less are likely to be therapeutically effective.

sulfadiazine and sulfathiazole. The bactericidal titer with sulfamethazine was 100 mg. per cent. Sulfamethazine administration led to complete clinical and bacteriologic cure within three days.

#### DISCUSSION

The method described is based on the assumption that the inhibitors contained in the broth do not affect the relative potency of the sulfonamides used in these tests, even if they do substantially lower the absolute potency. From the available evidence<sup>3, 7, 8</sup> it appears that the same amount of inhibitor affects sulfadiazine, sulfathiazole, and sulfapyridine equally. While such data are not available for sulfamethazine or Nu 445, our experience suggests that this is probably also the case for them.

Although the action of a sulfonamide on a given strain is stronger the simpler the composition of the medium,<sup>3, 7</sup> we observed that the marked differences in relative potency of the various sulfonamides on a given bacterial strain were exactly the same whether a simple synthetic medium or a broth was used (Table II). The difference in potency is of the order of one to two tubes. This observation indicates that the foregoing method is sufficiently accurate for routine laboratory work.

TABLE II. COMPARISON OF BACTERICIDAL TITERS IN MG. PER CENT OF STRAINS OF *ESCH. COLI* IN NUTRIENT BROTH AND IN THE SYNTHETIC MEDIUM OF KOHN AND HARRIS<sup>7</sup> IN VARIOUS SULFONAMIDES

STRAIN	NUMBER OF BACTERIA	MEDIUM (pH 7.2)	SD	SM	SMT	ST	PST	Nu 445
E. coli 1	2,000	Broth	750	750	250	100	250	500
		Synthetic med.	250	250	50	25	50	100
E. coli 2	4,000	Broth	1,000	750	500	100	500	750
		Synthetic med.	750	500	250	50	250	500
E. coli 3	3,000	Broth	1,000	1,000	750	1,000	1,000	250
		Synthetic med.	750	750	500	750	750	100
E. coli 4	4,000	Broth	500	500	100	250	500	750
		Synthetic med.	250	250	50	100	250	500

SD, sulfadiazine; SM, sulfamerazine; SMT, sulfamethazine; ST, sulfathiazole; PST, sulfathalidine; Nu 445, 3,4-dimethyl-5-sulfanilamido-isoxazole.

Similar comparative tests done with strains of *A. aerogenes*, *B. proteus vulgaris*, and *Ps. aeruginosa* gave similar results.

From the close correlation between results of the in vitro test and the therapeutic effect, two further conclusions may be drawn: (1) The choice of the sulfonamide should be made on the basis of the bactericidal and not the bacteriostatic titer. The gap between the two titers varies with nearly every strain. While they are occasionally identical, the bacteriostatic titer is often from one tube to three to four tubes removed from the bactericidal titer. (2) A sulfonamide exerting bactericidal action on a given strain in a concentration of 250 mg. per cent or lower is very likely to be therapeutically effective. If the bactericidal titer is 500 mg. per cent, the clinical action of the drug is questionable, but the drug should be tried if a more effective agent is not available. Sulfonamides with a bactericidal titer of 750 mg. per cent or higher are not therapeutically effective.

This study emphasizes the fact<sup>8</sup> that the sensitivity of different strains of a given species of bacteria is extremely variable. Some strains may be very sensitive and others completely resistant to the same sulfonamide.

#### SUMMARY

A simple method for determining the in vitro effect of various sulfonamides on a given bacterial strain is described. Clinical experiences are given showing the importance of the correct choice of sulfonamide.

The fact that a majority of the strains of a given species are usually sensitive to a sulfonamide does not assure a therapeutic effect in a given instance. Hence it is frequently necessary to determine the relative sensitivity of the strain responsible for the infection to a series of sulfonamides.

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# A MODIFIED ULTRAVIOLET SPECTROPHOTOMETRIC METHOD FOR QUANTITATIVE DETERMINATION OF BARBITURATES

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## INTRODUCTION

THE three drug depressions most commonly encountered in clinical medicine are due to the excessive absorption of alcohol, bromides, or barbiturates.

The first two of these agents may be determined readily, both qualitatively and quantitatively, by well-established clinical laboratory techniques. The qualitative detection of barbiturates also can be carried out with a fair degree of success by the Koppányi method providing sufficient blood or urine samples are available. However, until recently, quantification of the barbiturate levels in sera was not feasible since large quantities of blood were necessary.

Recently, spectrophotometric techniques for quantitative barbiturate determinations in sera have been proposed by others.<sup>1,2</sup> These techniques are extremely sensitive, so that an accurate barbiturate determination can be made on as little as 1 ml. of blood. The method developed in this laboratory has a distinct advantage over previous methods in that it can be applied directly to serum extracts with the elimination of interference due to serum and reagent blanks. We have used this procedure as a diagnostic aid in over fifty cases of barbiturate intoxication during the past year and have followed the blood levels of a number of these patients during their course of recovery in the hospital.

Determination of barbiturate sera levels aids the clinician in evaluating the relative effectiveness of various therapeutic measures he employs in his treatment of barbiturate intoxication.

## EXPERIMENTAL

*General Description of the Method.*—The barbiturate is extracted directly from serum in a semimicro continuous extractor using diethyl ether. The ether is evaporated and the barbiturate residue is brought into solution as the sodium salt using a borate buffer of pH 9.5. Optical density of the solution is determined by means of a Beckman spectrophotometer, and the amount of drug present is calculated using a standard curve prepared by a method which eliminates blank serum interference.

*Importance of the Influence of pH on the Absorption Spectra.*—The influence of pH on the absorption spectra of barbital and the 5,5 disubstituted barbiturates was reported by Elvidge,<sup>3</sup> Stuckey,<sup>4</sup> and more recently by Walker and co-workers.<sup>1</sup> We have confirmed these studies by measuring the absorption curves of six barbiturates in aqueous solution at varying pH values between 7.0 and 12.0. The barbiturates studied were Amytal, barbital, cyclo-barbital, Cyclopal, pentobarbital, and phenobarbital. The absorption curves for Amytal, which are similar to those of the other barbiturates tested, are shown in Fig. 1.

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The observation, as reported by Goldbaum,<sup>2</sup> that each barbiturate gives a characteristic absorption curve at pH 11 or greater suggested the possibility that the ratios of the optical densities at specific wave lengths could be used as criteria for the differentiation of the various barbiturate derivatives. The use of this means of identification is of little practical significance in clinical analysis, however, since the marked absorption due to blood or serum

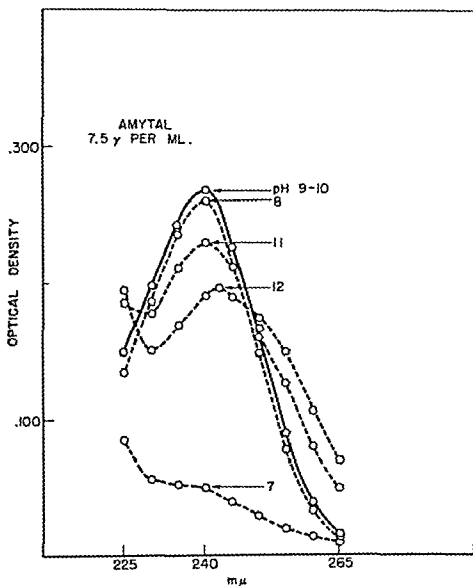


Fig. 1.—Absorption curves of Amytal in aqueous solution at varying pH values.

usually obscures not only the characteristic maximum but the other critical portions of the curve. The greatest amount of absorption per unit concentration is obtained between the pH values 9.0 and 10.0. Within this range of alkalinity all 5,5-disubstituted barbiturates develop peak absorption in the wave length range from 239 to 240 millimicrons. Although it is not possible to differentiate any one of the barbiturates by the shape of its absorption spectra in this pH range, the type of curve obtained with its steep slope is least likely to be obliterated by interfering serum blanks.

Thus, the optimum pH range for measurement of the absorption spectra of barbiturates was found to lie between 9.0 and 10.0. In the determination of drug levels in serum, unless the solution is carefully maintained in this range of alkalinity, variations may appear in the absorption curve which give erratic results. With reference to Fig. 1, it is evident that measurement of optical density at a pH of 11, rather than from 9 to 10, would result in only about 85 per cent recovery. For this reason we have used a borate buffer of pH 9.3 as the solvent in the determination of barbiturate absorption.

*Interference by Serum Blanks.*—Interfering absorption by ether or chloroform extracts of serum proved to be a problem of considerable difficulty. In experimental work



a blank serum specimen can be determined prior to the administration of the drug, and, provided no change occurs in the blank, a correction factor may be applied for the subsequent barbiturate determination. In clinical and forensic medicine, however, these blank samples are rarely available. Thus, the amount of absorption due to serum itself is unknown, and the accuracy of the quantitative determination of the drug is subject to a variable degree of error.

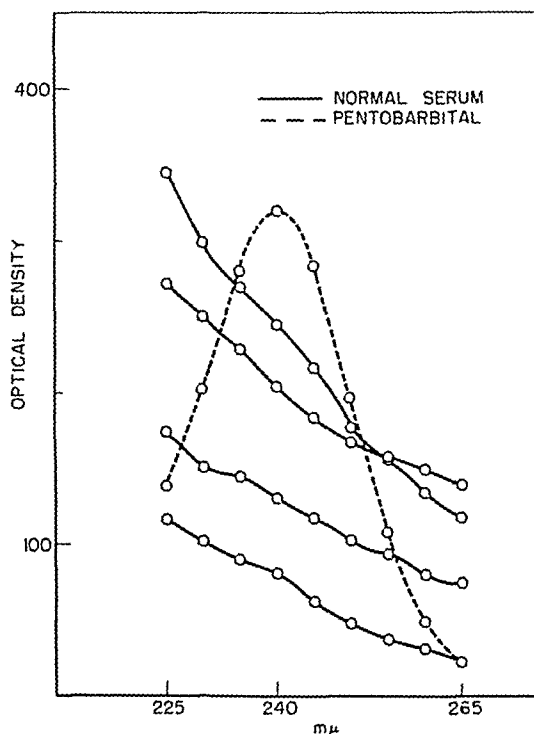


Fig. 2.—Absorption curves of ether extracts of normal human serum in borate buffer (pH 9.5). The absorption curve of pentobarbital (8 $\gamma$  per milliliter in borate buffer of pH 9.5) is superimposed to illustrate the importance of serum interference.

Goldbaum<sup>2</sup> has suggested an optical density value of 0.030 to be applied as a control blank in toxicologic analyses where no barbiturate-free samples of normal blood are available. This value was reported as the average for chloroform extracts of all normal blood samples measured at 255  $m\mu$  in 0.5N sodium hydroxide. Determinations were made in this laboratory of the optical densities of both ether and chloroform extracts of a series of 150 sera obtained from patients having had no history of recent drug ingestion. The optical densities of these "normal" sera were found to vary between 0.040 and 0.250 at wave length 240 millimicrons. Serum absorption measured at 255  $m\mu$  was only slightly less. The absorption curves of a few typical specimens of normal human serum are shown in Fig. 2, with an absorption curve of pentobarbital superimposed to illustrate the importance of blank serum interference. It is evident that no average blank value of any significance can be derived from these results.

Walker and associates have reported a method for the elimination of blank interference due to blood in which they employ an acid-alkaline shift.<sup>1</sup> The procedure is not applicable to direct extracts of blood or serum, as stated by Walker and confirmed by our experiments, and must be carried out on the filtrate of a tungstate-sulfuric acid protein precipitation. In so doing approximately 25 to 35 per cent of the barbiturate is unaccounted for, a factor which limits the accuracy of the method.

A satisfactory method of correcting for the interference of absorption due to serum and reagent blank<sup>4</sup> was developed in this laboratory by adapting a procedure described by Tannichiff.<sup>5</sup> The correction can be applied to extracts made directly from serum without preliminary protein precipitation. Fig. 3 illustrates an actual problem, demonstrating that the curve obtained from any clinical specimen (pentobarbital in serum)

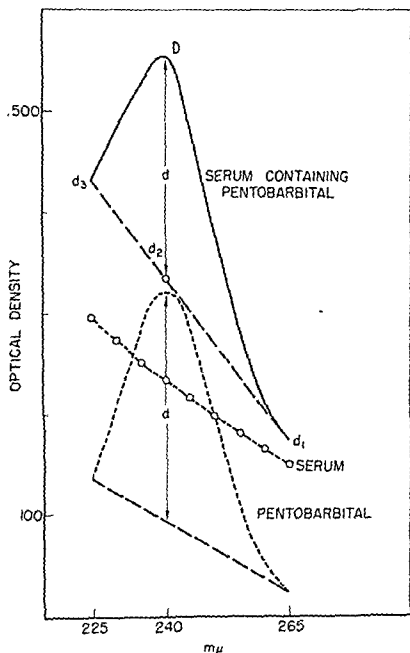


Fig. 3.—Absorption curves of ether extracts of serum and pentobarbital in serum, and standard pentobarbital curve. All absorption curves were determined in borate buffer at pH 9.5. The pentobarbital concentration was 8γ per milliliter.

is the additive effect of the blank serum curve plus the actual barbiturate curve. It is evident that the peak absorption, *D*, cannot be used as a measurement of drug concentration since an unknown fraction of its magnitude is due to serum absorption. The method we have adopted is based on the observation that the absorption curves of normal blank sera conform approximately to a straight line in the range of wave lengths from 225 to 265  $m\mu$  (Fig. 2). Thus, with reference to Fig. 3, if a base line is drawn between the optical density values  $d_1$  and  $d_3$ , a value, *d*, is obtained which serves as an adequate index for the amount of barbiturate present since it is directly proportional to drug concentration, and it is not significantly altered by serum blanks. This *d* value may be obtained either graphically

by plotting the three values,  $d_3$ ,  $D$ , and  $d_1$ , and drawing the base line  $d_3d_1$ , or by simple algebraic calculation using the equations:

$$d = D - d_2 \quad (A)$$

$$\text{where } d_2 = \frac{5}{8}d_3 + \frac{3}{8}d_1 \quad (B).$$

The derivation of (B) is as follows:

$$\frac{d_3 - d_1}{d_2 - d_1} = \frac{\lambda_3 - \lambda_1}{\lambda_2 - \lambda_1} \quad (C)$$

$$d_2 = d_3 \frac{(\lambda_2 - \lambda_1)}{(\lambda_3 - \lambda_1)} - \frac{d_1 (\lambda_2 - \lambda_1)}{(\lambda_3 - \lambda_1)} \quad (D).$$

Substituting wave length values in Equation (D) and solving

$$d_2 = d_3 \frac{(240 - 265)}{(225 - 265)} - \frac{d_1 (240 - 225)}{(225 - 265)} \quad (E)$$

which results in (B)

$$d_2 = \frac{5}{8}d_3 + \frac{3}{8}d_1 \quad (B).$$

A practical example will be cited to illustrate how the calculation may be applied (Table I). Following the regular extraction procedure the optical density was determined on, I, a blank sample of human serum, and III, the same serum to which had been added a known quantity of pentobarbital. II represents the standard absorption curve of the concentration of pentobarbital present in the serum. Table I includes the data as calculated algebraically using Equations (A) and (B). Fig. 3 illustrates the example graphically.

TABLE I. ILLUSTRATION OF ACCURACY OF CALCULATION OF BARBITURATE CONCENTRATION BY THE BASE LINE METHOD

WAVE LENGTH	SYMBOL	OPTICAL DENSITY		
		BLANK SERUM I	PENTOBARBITAL II	PENTOBARBITAL IN SERUM III
225 m $\mu$	$d_3$	0.295	0.137	0.432
240 m $\mu$	$D$	0.235	0.320	0.555
265 m $\mu$	$d_1$	0.150	0.023	0.173
	$d_2$	—	0.094	0.335
	$d$	—	0.226	0.220
$\gamma$ per ml.		—	8.0	7.8

By the use of this method the interference due to serum blanks is almost completely eliminated. In the foregoing example, the calculated value of barbiturate level is 97.5 per cent of the actual level. In practice, the accuracy of the method depends in part upon the nature of the blank serum absorption curve. The curve as a whole, from 225 to 265 m $\mu$ , does not always coincide exactly with the base line  $d_3d_1$ . This is not of great importance, however, since it is the amount of deviation in optical density between the serum curve and the base line at 240 m $\mu$  which is significant. This deviation is rarely more than shown in the example given, 0.006 optical density. The dependability of this correction for blank interference is reflected in the data contained in Table II.

#### PROCEDURE

1. One milliliter of serum is placed in the extractor, and from 5 to 10 ml. of distilled water are added, depending upon the volume of the extractor. Larger volumes of serum may

be used when available. Approximately 30 ml. of diethyl ether are placed in the receiving flask.\* The apparatus is assembled as shown in Fig. 4, the ether being evaporated in a water bath regulated between 50 and 55° C. In the event that emulsions are formed at the interface, they are readily removed by addition of a few crystals of anhydrous sodium sulfate. When

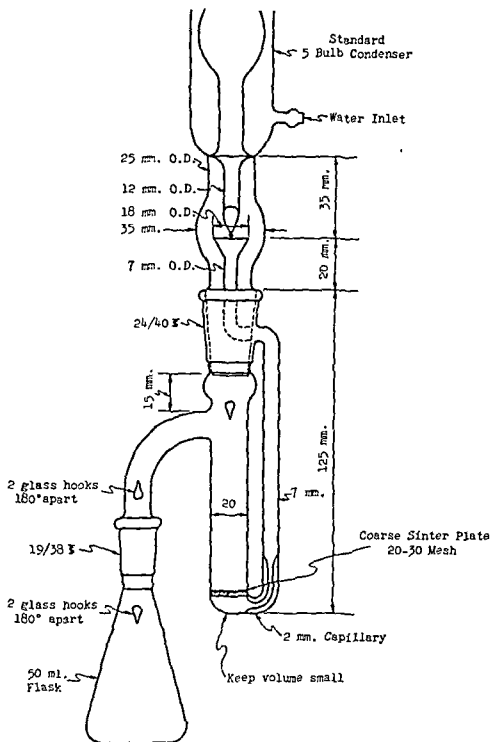


Fig. 4.—Diagram of semimicro extraction apparatus.

extraction is complete (fifteen to sixty minutes) the ether may be evaporated into a clean extractor and saved for redistillation. The receiving flasks containing the barbiturate residue must be completely dry before addition of the borate buffer. This is best accomplished by placing them in a drying oven for a few minutes.

\**Reagents.* Diethyl ether, technical grade: Washed with distilled water, treated with ferrous sulfate, dried with anhydrous calcium chloride, and redistilled, preferably over metallic sodium.

Borate buffer. 0.2M. boric acid C.P.

0.2M. potassium chloride, C.P.—Solution A

0.2M. sodium hydroxide, C.P.—Solution B

prepared according to Peters and Van Slyke<sup>4</sup> to pH 8.5.

*Apparatus.* Semimicro continuous extractor with condenser and receiving flask (Fig. 4) Water bath with thermostatic control ranging from 50 to 55° C. Beckman Model DU Ultra-violet Spectrophotometer with 1 cm. quartz absorption cells.

TABLE II. RECOVERIES OF BARBITURATES ADDED TO BLANK SERUM  
(LEVELS WERE CALCULATED BY THE BASE LINE METHOD)

NUMBER OF DETERMINATIONS	MICROGRAMS ADDED	AVERAGE RECOVERY* (PER CENT)
11	14.4	92.2 ± 2.75
14	20.0	98.5 ± 1.95
12	75.0	101.2 ± 1.28
10	100.0	95.7 ± 1.81

\*This includes one standard error of the mean.

2. When dry, the barbiturate residue is brought into solution with an accurately measured quantity of borate buffer (pH 9.5). A volume of 2 ml. or more may be used, depending upon the concentration of drug present.

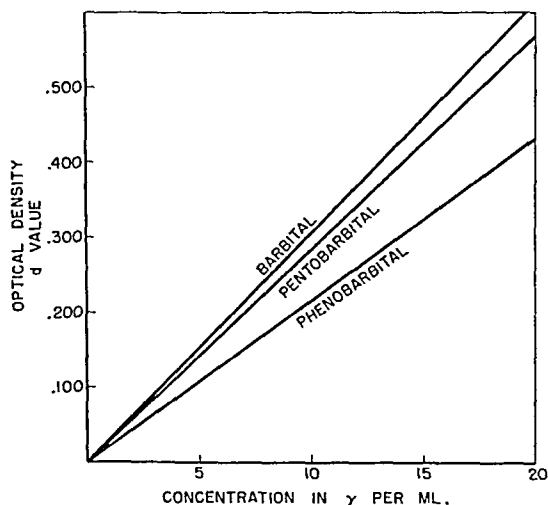


Fig. 5.—Standard concentration curves of a few representative barbiturates. The optical density is measured in  $d$  units calculated from Equations A and B.

3. With the barbiturate present in the alkaline form in the pH range from 9.0 to 10.0, absorption is determined at three wave lengths, 225, 240, and 265  $m\mu$  ( $d_s$ ,  $D$ , and  $d_i$  respectively), and the  $d$  value is calculated by using Equations (A) and (B).

4. Barbiturate concentration in the borate buffer is determined by reference to a standard curve (Fig. 5).

The value may be expressed as milligrams per cent in the sera according to the equation:

$$\text{Mg. per cent of barbiturate} = \frac{\gamma B}{10 S}$$

where  $\gamma$  = micrograms of barbiturate read from the standard curve

$B$  = milliliters of borate buffer

$S$  = milliliters of sample.

*Accuracy of the Method.*—Table II includes data from a series of experiments to determine the accuracy of the method. Known amounts of barbiturates were added to normal sera (beef, horse, and human) of unknown optical density levels. The samples were determined according to the procedure outlined in the preceding section with 1 ml. serum and in all instances the base line method was used to determine the drug level. Amytal, barbitol,

pentobarbital, phenobarbital, and Seconal were the representative barbiturates included in the study. No significant difference between the recovery of the five barbiturates was noticed.

#### DISCUSSION

*Base Line Method of Calculation.*—The three wave lengths, 225, 240, and 265  $m\mu$ , were empirically chosen since this combination of wave lengths resulted in the greatest  $d$  value per unit concentration. For the actual barbiturate curve (Fig. 3), the farther removed the values  $d_3$  and  $d_1$  are from  $d_2$ , the greater the  $d$  index. There are, however, certain limiting factors: at wave lengths less than 225  $m\mu$  there is a sudden steep rise in serum absorption as barbiturate absorption falls off. Beyond 265  $m\mu$ , although serum absorption is gradually becoming less, the drug absorption also decreases. The wave length 240  $m\mu$  is the logical place for the  $D$  absorption since peak absorption occurs here.

*Interfering Substances.*—There are three types of compounds of clinical importance which might cause interference in the barbiturate determination.

1. *Sulfonamides:* Although alkaline solutions of sulfonamides elicit absorption in the critical range of the barbiturate spectrum, they may be almost completely eliminated when present in serum by the process of extraction. By carrying out the extraction procedure at the pH of serum, only negligible interference by sulfonamides was obtained with levels as high as 10 mg. per cent in serum. The absorption curve of the small amount of sulfonamide which is extracted is such that at 240  $m\mu$  there is no deviation of the curve from the  $d_3d_1$  base line. The following sulfonamides were tested at concentrations of 10 mg. per cent and were not found to interfere with the determination: sulfanilamide, sulfadiazine, sulfaguanidine, sulfapyridine, sulfamerazine, sulfathiazole.

2. *Salicylates:* The salicylates give absorption curves with two maxima, one in the critical range of barbiturate absorption and the other beyond the limit where absorption of the non-thio type of barbiturate occurs. Salicylates can be identified easily by their characteristic absorption curves and consequently should not be mistaken for barbiturates. There is at present no satisfactory method of eliminating their interference.

3. *Products of Barbiturate Metabolism:* In the metabolism of the nonsulfur-containing barbiturates the splitting of the malonyl urea ring yields a compound which does not produce interfering absorption in the ultraviolet range. It is possible that alterations of the barbiturate other than breakdown to a ureide could yield metabolic products which would contribute to the absorption spectrum. Whether or not these compounds would be extracted by our method and whether or not their absorption curves could be differentiated from those of the parent compound are problems which are in need of further investigation.

In cases of barbiturate intoxication where the identity of the barbiturate is not known, there is an inherent error in all spectrophotometric methods, since barbiturates differ to some degree in their molecular extinction coefficients. From a practical standpoint this difference is not of great importance. It has been our practice when the ingested barbiturate was not

known or could not be qualitatively identified to express the sera level in terms of pentobarbital, since the  $d$  values for this compound are closest to the mean of the commonly encountered barbiturates.

#### SUMMARY

A method for quantitative barbiturate analysis has been developed which has the following advantages.

1. The interference due to absorption of reagent and serum blanks is eliminated by means of a graphic solution or a simple algebraic calculation.
2. Careful regulation of pH in determining ultraviolet absorption increases the accuracy of the determination.
3. Samples as small as 1 ml. of serum may be extracted directly without preliminary protein precipitation.
4. There is a considerable economy of glassware and reagents involved as well as ease of extraction.

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## FAT DETERMINATION IN FECES USING MOJONNIER EXTRACTION FLASKS

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**I**N FAT balance studies the methods commonly employed for the determination of the fat in feces have not been particularly satisfactory. The procedure of drying the fecal specimens is cumbersome and time consuming, and an error is introduced because during the drying and grinding process part of the feces sticks to the walls of the receptacles. Extraction of fat with the Soxhlet apparatus is difficult where the water pressure is not constant and the water is not sufficiently cold to condense the ether vapors. This latter feature is particularly significant in warm climates. Extraction methods employing wet feces are simple and efficient, but it is difficult to avoid emulsification in extraction with alcohol and ethyl ether, hence centrifugation is often necessary.

### METHOD

An adaptation of the Roesse-Gottlieb fat extraction method applied to wet feces and using Mojonnier extraction flasks\* was suggested by Hilda F. Wiese, of the Department of Pediatrics.

The feces are washed with distilled water into a tared jar or a Waring Blendor for mixing. Water is added to make the sample sufficiently fluid to be drawn into a pipette. The total weight of feces and water is taken. After thorough mixing either in the Waring Blendor or with an electric stirrer, which can be inserted directly into the weighed jar, 3 to 8 ml. aliquots are withdrawn and transferred directly into the bottom chamber of the weighed Mojonnier fat extraction flasks. The extraction flasks are weighed directly on an analytic balance. If the total fat excreted is to be reported on the dry basis, separate aliquots are measured out for the determination of total solids.

For the extraction a few drops of concentrated HCl are added, then 12 to 15 ml. 95 per cent alcohol. The flasks are tightly stoppered and shaken for thirty seconds. Then 20 ml. ethyl ether are added; the flasks are stoppered and shaken vigorously for sixty seconds. This is followed with 25 ml. petroleum ether and the shaking is continued for sixty seconds. The flasks are then allowed to stand ten to fifteen minutes for complete separation of the ether layer. The Mojonnier fat extraction flasks are so constructed that the ether layer can be poured off directly into weighed containers for evaporation of the solvents without danger of contamination from the aqueous layer. (See Fig. 1.) The extraction procedure is repeated using 5 ml. 95 per cent alcohol, 15 ml. ethyl ether, and 15 ml. petroleum ether. If the fat content of the feces is very high, it may be necessary to make a fourth extraction. Just before the last ether layer is poured off, sufficient water is added to fill the bottom chamber, so the ether layer can be removed completely. The ether extracts are evaporated on the water bath; the flasks are dried in a desiccator and weighed. With the given proportions of alcohol and ether, emulsions rarely occur.

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\*The Mojonnier fat extraction flasks are manufactured by Mojonnier Brothers Company, 4601 West Ohio Street, Chicago 44, Ill.



However, if emulsification results, this can usually be broken by the addition of a small amount of alcohol. Also, it is sometimes desirable to re-extract the fat residues with petroleum ether alone. These can be filtered readily through fat-free cotton inserted into a small funnel.

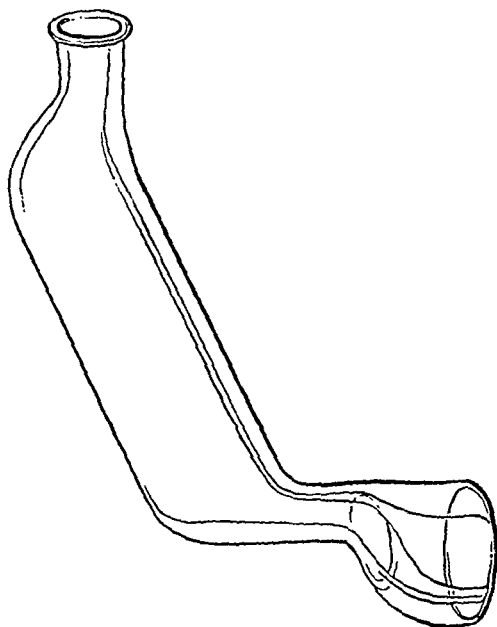


Fig. 1.—Mojonnier flask used for fecal fat extraction.

#### RESULTS

In fifty consecutive duplicate determinations, forty-three showed a difference of less than 2.5 per cent. Two of the samples contained less than 1 per cent fat and the differences here were higher (3.3 and 6.1 per cent). These specimens were obtained from infants who were maintained on a skimmed milk mixture. In the other five instances, differences of 3.3 to 5.1 per cent were obtained. These latter determinations were made at the beginning of the study, and the maximum variation of 5 per cent in duplicate samples seemed satisfactory, hence they were not repeated.

The method described offers several advantages. There is little loss of feces in the preparation and transfer of the material. The extraction can be completed in a very short time and emulsification rarely occurs. Duplicate samples agreed within narrow limits, except in two instances where the fat content was less than 1 per cent of the sample.

# THE SIGNIFICANCE OF CHOLESTEROL VARIATIONS IN HUMAN BLOOD SERUM

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IT HAS been pointed out that the literature dealing with serum concentrations of lipids and cholesterol is filled with conflict.<sup>1, 2</sup> These inconsistencies appear to be accounted for by differences in technique and the unreliability of certain methods now widely used. This subject has been reviewed adequately elsewhere.<sup>3, 4</sup> Accordingly, a modification of the Sperry-Schoenheimer method<sup>4</sup> has been employed for cholesterol, as developed by Chaney and Lovell.<sup>5</sup> This modification has been checked against the original Sperry-Schoenheimer method and also against other procedures in wide use. The results again emphasize the need for adhering to the fundamental principles of the Sperry-Schoenheimer procedure and demonstrate that wide variations in results are likely to occur in other methods which do not adequately control the Liebermann-Burchard color reaction. The modification of Chaney and Lovell appreciably curtails the time required for the procedure and reduces the complexities of the original Sperry-Schoenheimer method while maintaining its accuracy.

The need for standardization and perfection of such technique has been even more emphasized by the recent renewal of interest in and investigations into the role of fat metabolism in the production of arteriosclerosis and in particular coronary thrombosis. Recent studies<sup>2</sup> suggested that serum cholesterol levels are subject to variations under certain conditions in the same subject at different time periods. We therefore undertook an investigation into these circumstances of variation and present the following results.

The additional value of such an inquiry would be in supplying further criteria on which to evaluate the role of certain lipotropic agents, such as choline, inositol, etc. These have been recently employed both experimentally and clinically as "decholesterizing" agents in arteriosclerosis.<sup>6-9</sup> Changes in serum cholesterol values were reported<sup>8</sup> following the use of these agents.

## CLINICAL MATERIAL

Two or more fasting serum cholesterol tests were made at intervals in 161 subjects who were divided into four groups. Group A consisted of thirty-two normal individuals who were free of any clinical symptoms or demonstrable evidence of any illness. These subjects served as the normal controls. Group B was selected as a control group comprising thirty-one patients who had varied illnesses such as chronic peptic ulcer, chronic

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nephritis, chronic nonspecific ulcerative colitis, cirrhosis of the liver, chronic, rheumatoid arthritis, and essential hypertension. No patient in this group had known evidence of coronary occlusion or angina pectoris. Group C consisted of fifty patients who had had recent coronary occlusions (within a six-month period of time) but who received no specific medication or lipotropic agents, except in a few instances where maintenance doses of digitalis or Mercuhydrin had been prescribed. Group D comprised forty-eight patients who had been on 6 Gm. daily doses of choline bicarbonate for variable periods of time and who had suffered a recent coronary occlusion within six months of the institution of choline therapy. As in Group C, several patients were on maintenance doses of digitalis or Mercuhydrin.

## RESULTS

*Group A.*—Thirty-two normal subjects were studied whose ages ranged from 13 years to 72 years, the average age being 35 years. Twenty were male subjects; twelve were female. The time interval between the first cholesterol determination and the second (which was the last in most instances) ranged from eight weeks to sixty-four weeks, the average time interval being thirty-six weeks. As shown in Fig. 1, the average initial serum cholesterol was 202 mg. and the average final serum cholesterol was 200 milligrams. The variations between these two ranged from 0 to 38 mg., with an average variation of 11 milligrams. The normal serum cholesterol values are from 140 to 220 milligrams. Thus Fig. 1 illustrates the constancy of cholesterol levels in normal controls, when two or more determinations separated by a considerable period of time (one to sixty-four weeks) are made on the same individual. It will be noted that the increase or decrease in level is slight, less than 20 mg. per cent (or about 10 per cent of the total) in nearly all instances.

*Group B.*—This group consisted of thirty-one patients with various diseases as noted, excluding known illness due to coronary artery disease. The ages ranged from 24 years to 62 years, the average being 46 years. Twenty patients were men; eleven were women. As shown in Fig. 2, miscellaneous patient controls, the average initial cholesterol was 279 mg. and the average final cholesterol was 280 milligrams. The intervals of observation ranged from one week to twenty-six weeks, the average being nine weeks. In this group were twelve patients whose serum cholesterol varied less than 10 per cent between the first and last determinations, the average amount of cholesterol variation being 23 milligrams. There also were ten patients whose serum cholesterol values showed substantial decreases, on an average of 83 mg. between the first and last determinations. There were an additional nine patients whose serum cholesterol increased on an average of 73 mg. between the first and last determinations. In Fig. 2, describing this miscellaneous series of conditions, some involving elevated cholesterol values, but in which coronary artery disease was absent, the tendency for greater fluctuations to occur is evident from the graph, and increases or decreases up to 100 mg. or more occur as frequently as lesser deviations.

*Group C.*—Group C consisted of fifty patients who had had recent coronary artery occlusions (coronary occlusion controls), Fig. 3, within six months prior to the study. Their ages ranged from 38 years to 80 years, the average being 62 years. Forty patients were men; ten patients were women. The interval

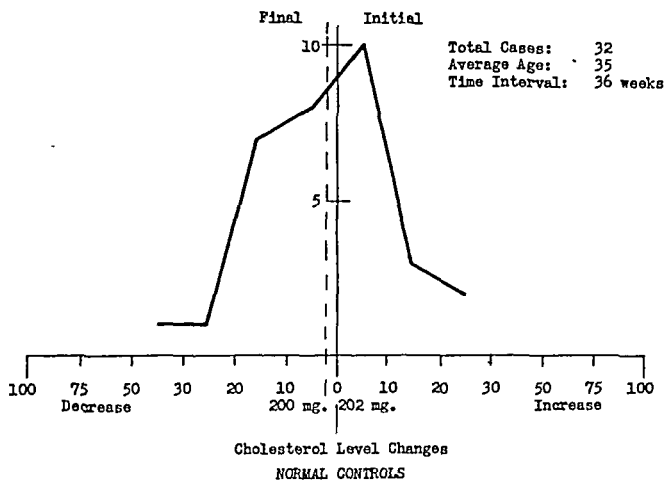


Fig. 1.

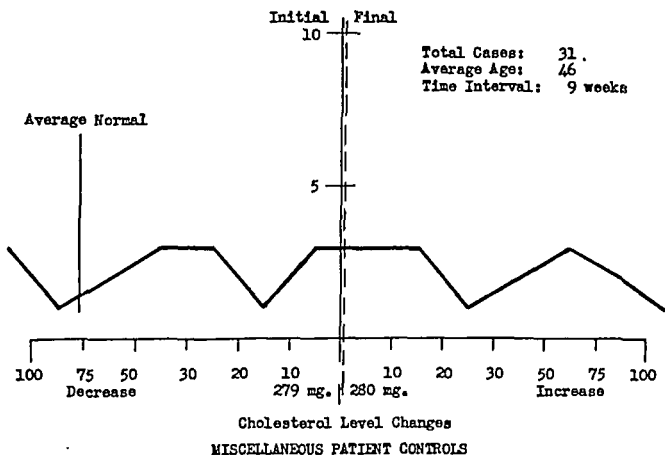


Fig. 2.

range between initial and final cholesterol determinations was from one week to sixty weeks, the average being fourteen weeks. In this group there were three further subgroups. Ten patients had less than 10 per cent variations in serum cholesterol from first to last determinations, the average variation being 26 milligrams. Nineteen patients showed substantial interval cholesterol decreases with an average decrease of 69 milligrams. Twenty-one revealed interval

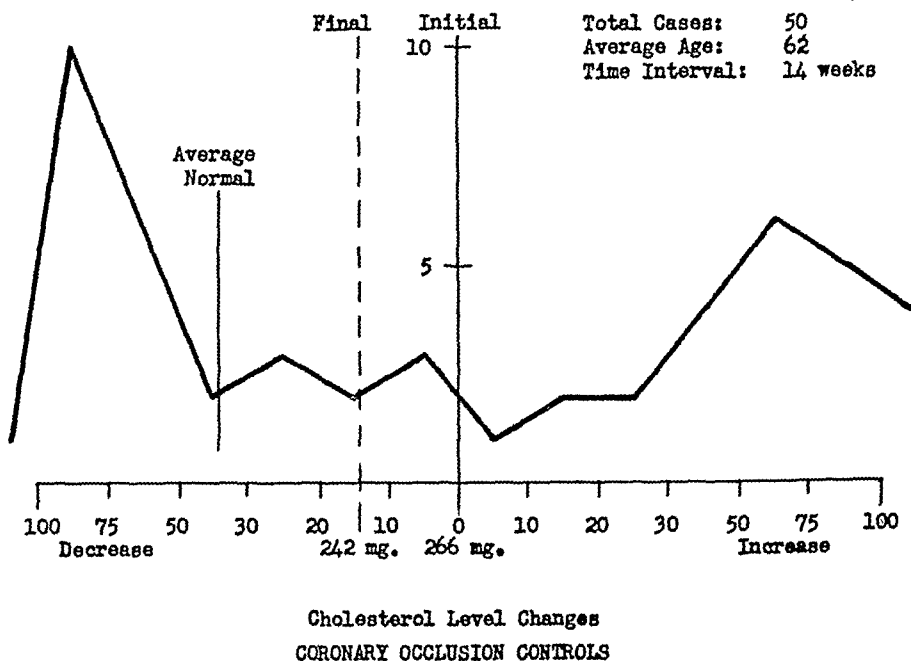


Fig. 3.

cholesterol increases with an average of 83 mg. increase. Fig. 3 (coronary occlusion controls) reveals that the average initial serum cholesterol was 266 mg. and the average final serum cholesterol was 242 milligrams. In acute coronary occlusion, which condition is frequently associated with elevated blood cholesterol, as has been shown previously,<sup>2</sup> there is a more marked tendency to fluctuate. Here it is noted that the majority of cases show a marked difference in level from an initial determination to a succeeding one and that this difference may as frequently be an increase as a decrease.

*Group D.*—This group consisted of forty-eight patients who had had recent coronary artery occlusion within six months prior to testing and treating, most of whom had received 6 Gm. of choline bicarbonate daily. This was given in three divided daily doses, each containing 2 Gm. (coronary occlusion—choline therapy, Fig. 4). The ages ranged from 29 years to 80 years, with an average age of 59 years. Forty-two patients were men and six patients were women. The interval range between the first and last cholesterol while on choline therapy was from one week to sixty-seven weeks, the average being twelve weeks. This

group had three further subgroups. One consisted of thirteen patients who showed less than 10 per cent interval variation in serum cholesterol values. The average variation was 5 milligrams. Eighteen patients revealed an average interval decrease of 72 mg. while on choline therapy. Seventeen patients showed an average interval increase of 98 mg. on choline therapy. Fig. 4 further shows how these fluctuations render more difficult the interpretation of the effect of

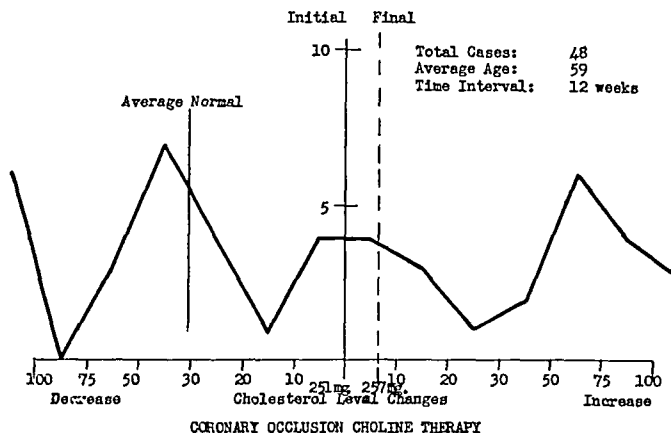


Fig. 4.

choline feeding on cholesterol levels. However, by graphing the results on this series (Fig. 4) in the same manner, it is apparent that no significant change can be attributed to the choline which is not already accounted for by the fluctuations inherent in the disease.

#### DISCUSSION

Confirmation is again made of Sperry's observation that in normal health the blood serum cholesterol is maintained at a constitutional level characteristic for the individual and from which significant alterations are not prone to take place. It is of interest to note here that in two of the normal control subjects, wider variations in serum cholesterol were found than in the other subjects. Upon careful interrogation, it was elicited that one subject, aged 41, had had a severe attack of infectious hepatitis eighteen years previously. Liver function tests in this individual had shown moderate impairment up to five years following recovery but had not been repeated, since the subject felt himself to be in good health. After the serum cholesterol determinations had been made and were found to be more variable than in the other normal subjects (a variation of 29 mg.), liver function tests were repeated and showed again low-grade impairment of liver function tests.

The other normal control subject, age 42, who revealed a 38 mg. variation, was closely questioned and was found to have a brother who had died recently of an acute coronary occlusion with hypercholesterolemia, one sister who had had a coronary artery occlusion, and parents who had died of coronary occlusions. A familial susceptibility or hereditary cholesterol metabolism disorder associated with coronary artery disease may be a possibility in this case.<sup>13</sup> Our control series of fifty cases of coronary thrombosis (Group C) confirms the findings of Steiner and Domanski<sup>11</sup> who also found that the serum cholesterol level in patients with coronary arteriosclerosis is variable and subject to wide fluctuations. Our finding of wide serum cholesterol fluctuations in miscellaneous diseases is in agreement with the report just published by Astrup.<sup>12</sup> The Group D patients with coronary artery occlusions who were under choline therapy revealed the same type of fluctuations of serum cholesterol observed in the control group of noncholine-treated coronary artery occlusion cases. That is to say, there were three similar subgroups, one showing 10 per cent fluctuation in serum cholesterol values on choline treatment, another showing significant increases in serum cholesterol values on choline treatment, and the other showing significant decrease under therapy.

It has been demonstrated that the liver exerts the chief control over cholesterol metabolism, as well as fat metabolism.<sup>1</sup> It also has been established that the liver plays the main role in the synthesis, distribution, and regulation of cholesterol in the blood stream. It is therefore possible that abnormal variations or instability in the serum cholesterol levels encountered in Groups B and C, comprising coronary thrombosis and certain other diseases, indicate an impairment in the function of the liver regulating cholesterol metabolism in these diseases.

#### SUMMARY AND CONCLUSIONS

1. In a series of thirty-two normal subjects, the constancy of blood serum cholesterol levels was reaffirmed over prolonged test periods using a modification of the Sperry-Schoenheimer serum cholesterol procedure.

2. Wide variations in serum cholesterol values were found in a group of thirty-one patients with miscellaneous diseases.

3. Marked fluctuations in serum cholesterol values were observed in a series of fifty patients who had recently experienced a coronary artery thrombosis.

4. Analogous wide fluctuations in serum cholesterol values were found in a series of forty-eight patients who had recently experienced a coronary artery thrombosis and ingested 6 Gm. of choline daily. These fluctuations rendered it impracticable to determine whether choline effected a reduction or increase in serum cholesterol levels.

5. It is suggested that variations or instability in serum cholesterol exceeding 15 per cent when determined by the Sperry-Schoenheimer procedure or a proved modification thereof in an individual presumed to be normal may possibly indicate a systemic disorder or latent illness.

6. It is suggested that if the Sperry-Schoenheimer procedure or a proved modification thereof shows abnormal variations in serum cholesterol, these may be due to a disturbance in the function of the liver regulating cholesterol metabolism.

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# IRON METABOLISM

## ERYTHROCYTE IRON TURNOVER

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THE hemoglobin cycle in man is particularly accessible to studies employing radioactive isotopes of iron. The incorporation of radioiron into circulating hemoglobin or the disappearance of tagged cells from circulation may be measured. Such studies have necessarily been of short duration. In the turnover of the red cell mass, iron liberated from hemoglobin is passed on to other tissue and serum proteins (Fig. 1) and rapidly rerouted to the bone marrow. Once the radioiron reaches a state of equilibrium in the circulating red cell mass, the level of radioactivity may remain relatively constant for months or years. In the present study it has been possible to isolate the erythrocyte portion of the hemoglobin cycle, so that the survival of a population of red cells of approximately the same age might be determined.

The assumption has been made on the basis of *in vitro* studies<sup>1, 2</sup> that circulating red cells neither take up nor give off iron. The reticulocyte, however, has recently been shown to take up iron and synthesize heme.<sup>3</sup> These studies have allowed further observations *in vivo* and over a long period of time regarding the question of iron exchange between red cell and plasma.

Certain observations have previously been made regarding the breakdown and reutilization of hemoglobin iron. In studies on blood preservation, donors were prepared by injecting radioiron and obtaining blood after the iron had been incorporated in the red cell mass. The disappearance rate of these tagged erythrocytes from the blood stream of the recipient was measured over a period of twenty-four to forty-eight hours.<sup>4-7</sup> Thereafter, the radioiron was rapidly incorporated into new red cells so that in the ensuing days the level of radioactivity approximated that originally present. If red cell destruction occurred more gradually, no drop in the circulating radioactivity level was observed. This efficient reutilization of broken down hemoglobin iron has been commented on by several investigators as indicating that iron so liberated from hemoglobin falls into a small labile reserve which is rapidly recircuited through the hemoglobin cycle.<sup>8, 9, 10</sup> It has been shown that this utilization of radioiron for hemoglobin production may be largely prevented under two circumstances:<sup>11</sup>

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first, when the bone marrow is producing little or no blood, and second, when the iron stores are greatly enlarged. Advantage was taken of this in the choice of recipients for our studies. Subjects were selected who for one of these reasons should be unable to reutilize the radioiron liberated from tagged erythrocytes. It would then be possible to follow the intraerythrocytic radioiron through the period of time that the red cell remained in the circulation, provided no free exchange of iron occurred.

## PROTEIN-IRON COMPLEXES OF THE HEMOGLOBIN CYCLE

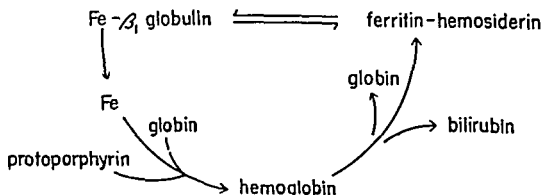


Fig. 1.—In the serum, iron is bound to a beta<sub>1</sub> globulin which transports iron from one tissue to another within the body. In the marrow the iron is stripped from the beta<sub>1</sub> globulin by the developing red cell and synthesized along with pyrol pigment and globin into hemoglobin. As hemoglobin, the red cell iron would appear to remain within the erythrocytes until the stroma is broken down. At this time, the pigment is degraded into its component parts and the iron may reappear in the serum bound to the transport globulin or may remain in the tissues incorporated in one of the iron storage proteins, ferritin or hemosiderin.

### MATERIALS AND METHODS

Single isotopes of Fe<sup>55</sup> and Fe<sup>59</sup> obtained by cyclotron bombardment at the Massachusetts Institute of Technology were employed in both human and animal studies. Radioactive iron containing both isotopes Fe<sup>55</sup> and Fe<sup>59</sup> with a specific activity of 20 and 80 microcuries per milligram of iron, obtained from Oak Ridge, was used in some animal experiments. Radioactive donors were prepared as previously described.<sup>2</sup> Tagged blood for transfusion was drawn into acid citrate dextrose preservative and given immediately to the recipient except in one instance in which the effect of storage was under study. The circulating level of radioactivity approximately twenty minutes after blood was given to the recipient was determined and taken to represent 100 per cent of the transfused cells. In two instances, however, where subsequent levels were significantly higher, the latter values were assumed to represent 100 per cent. The blood volume as determined by T1824 dye was compared with the cell volume as determined by dilution of the transfused tagged cells. Subsequent blood samples were taken into calibrated centrifuge tubes over a period of 150 days. Hematocrits were determined on an aliquot by the Wintrobe method. The subsequent wet ashing of samples, addition of carrier iron, precipitation, electroplating, and counting have been described previously.<sup>12</sup>

### EXPERIMENTAL DATA

*I. Animals.*—Dog S-FD90 was given 0.2 mg. Fe<sup>55</sup> ( $1 \times 10^6$  counts per minute) intravenously and the subsequent incorporation of the iron into the circulating red cell mass was determined (Fig. 2). At fifteen days 75 per cent

of the injected iron was found in the peripheral blood.\* On the twenty-seventh day and until the 108th day injections of nonradioactive iron in the form of ferrous-ascorbate gelatin† totalling 4,170 mg. were given intravenously. Throughout the period of observation the dog remained in good condition.

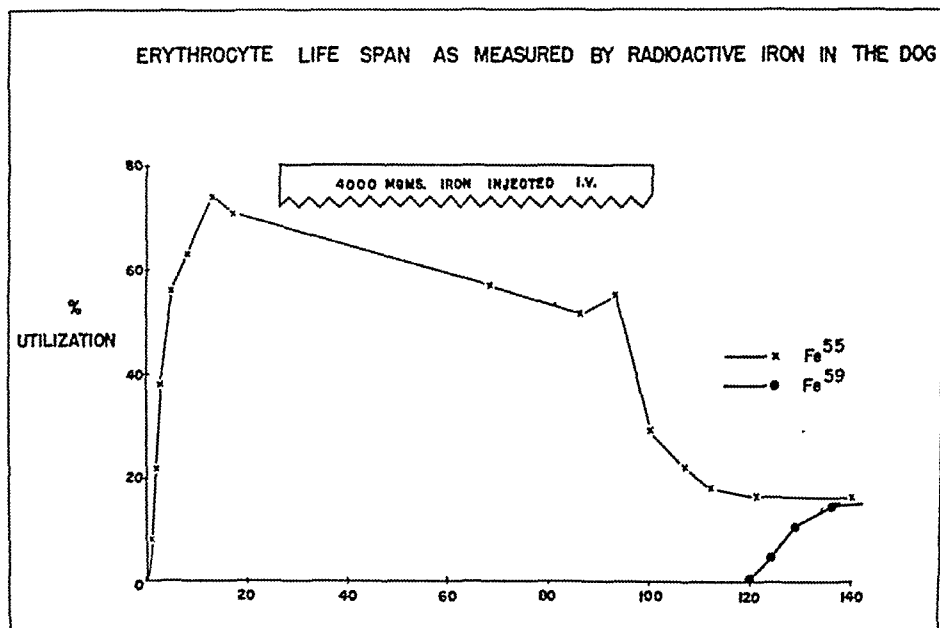


Fig. 2.—The initial utilization of Fe<sup>55</sup> in a normal dog is shown. Circulating radio levels were followed during a period of 100 days while nonradioactive iron was injected intravenously. A subsequent utilization curve with Fe<sup>59</sup> is shown. The ordinate is expressed in days.

There was a gradual drop in hemoglobin from 18 to 15 grams but no change in cell indices, reticulocytes, or in the leucocyte picture. In Fig. 2 is shown the level of circulating radioactivity. At 120 days 0.5 mg. of Fe<sup>59</sup> ( $1 \times 10^6$  counts per minute) was injected intravenously. Sixteen per cent of this iron appeared in the red blood cell mass at the end of two and a half weeks.

In two further experiments dogs were placed on corn grit and iron diets which had previously been shown to produce large iron stores in animals.<sup>13</sup> These dogs received red cells prepared by injecting radioiron in a single donor dog one week previously. A similar rapid decline in circulating radioactive levels was observed in these dogs, in the neighborhood of 100 days. The red cell life was calculated from the time 50 per cent of the tagged cells had appeared in the blood of the donor dog to the time 50 per cent of the circulating radioactivity had been lost from the blood of the recipient dogs. In Dog J-FD219 the erythrocyte life span was 106 days and the average survival in Dog C-FD218 was 109 days. These dogs showed no significant hematologic

\*The dog's blood volume was determined by T1824 dye and the per cent circulating radioactivity calculated as previously described<sup>11</sup> according to the formula: per cent activity = counts per cubic centimeter red cells  $\times$  cubic centimeter circulating red cells divided by the counts injected.

†This iron preparation was obtained through the courtesy of the Knox Gelatine Protein Products, Inc., Camden, N. J.

change through the period of experimentation. The pathologic examination of both dogs revealed extensive deposits of hemosiderin and greatly increased tissue iron by analysis.

### LIFE SPAN OF ERYTHROCYTES TAGGED BY RADIOIRON

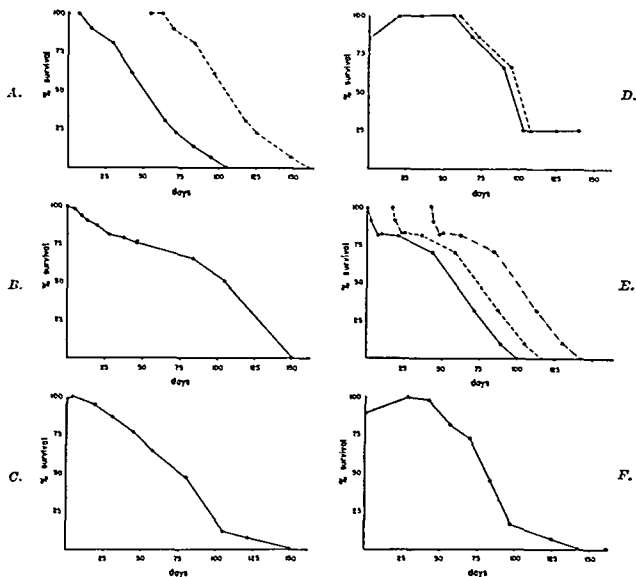


Fig. 3.—The solid line represents the actual radioactivity levels in the circulating blood of the recipients. The dotted line in A, D, and E allows for the added period of red cell life in the donor. In E, the third line represents the additional period of blood storage.

A, Sixty-day-old normal cells to patient with aplastic anemia.

B, Pernicious anemia reticulocytes to patient with aplastic anemia.

C, Iron-deficient reticulocytes to patient with aplastic anemia.

D, Iron-deficient erythrocytes to a patient with hemochromatosis.

E, Twenty-one-day-old blood stored for twenty-four days transfused to a patient with Cooley's anemia.

F, Iron-deficient reticulocytes transfused to a patient with Cooley's anemia.

**II. Human Subjects.**—Six populations of radioactively tagged erythrocytes were followed in recipients chosen because of their large iron stores and/or impaired bone marrow function. There follows in each instance a characterization of the blood given, the age of the red cells given, the hematologic status of the recipient, and the radioactive survival curves as determined by measuring levels of radioactivity in the recipient blood. The data are plotted in Fig. 3.

A: Five hundred cubic centimeters of blood were drawn from a young man who had been given radioiron ( $\text{Fe}^{55}$ ) sixty-five days previously. The donor's red cells were normal in appearance and measurements showed a mean

corpuscular volume (MCV) of 85, a mean corpuscular hemoglobin (MCH) of 32, and a mean corpuscular hemoglobin concentration (MCHC) of 35. The recipient W-FD129 was a girl of 6 years with aplastic anemia who had been transfused every six to eight weeks since the age of 2 months. Preceding the transfusion her blood showed a hemoglobin of 5 grams, red count 1.8 million, and hematocrit 16 (MCV 108, MCHC 28, MCH 31) and no reticulocytes in 1,000 red blood cells. The subsequent decline in circulating radioactivity levels is shown in Fig. 3, A. The projected life span of the red cells, allowing for the time they had circulated in the donor, is shown by the dotted line.

*B:* The same recipient, W-FD129, was transfused one month later with 250 c.c. of blood tagged with  $\text{Fe}^{59}$ . The donor in this case, G-FD131, had classical pernicious anemia and had received liver six days previously and radioiron four days before the blood was drawn. The transfused blood had a MCV of 130, MCH 37, and MCHC 35. There were 31.9 reticulocytes. In the blood fractionated by albumin flotation<sup>14</sup> the distribution of radioactivity corresponds to the distribution of reticulocytes (Table I). These data would indicate that the isotope is found almost exclusively in the reticulocyte fraction and that, in effect, tagged reticulocytes are being transfused. Subsequent survival of this blood is shown in Fig. 3, B.

*C:* Recipient W-FD129 was used for a third study after all previously administered radioactivity had disappeared from her circulating blood. Donor SL-FD262 had typical untreated polycythemia vera. The donor's blood showed a hemoglobin of 20.1 grams with slight microcytosis and hypochromia (MCV 75, MCH 23, MCHC 31). Five hundred cubic centimeters of blood were drawn on the third day after injection of radioiron. Evidence that these tagged cells were comprised chiefly of reticulocytes is shown in Table I. The determinations of circulating radioiron levels in the recipient are shown in Fig. 3, C.

*D:* Five hundred cubic centimeters of blood were drawn from the same donor, SL-FD262, on the tenth day following injection of radioiron. This blood was transfused to recipient R-FD108 who had hemochromatosis confirmed by

TABLE I

IRON DEFICIENT BLOOD	COUNTS/UNIT CELLS	RETICS (%)
Top fraction	2,900	10.8
Middle fraction	920	2.9
Bottom fraction	500	1
PERNICKIOUS ANEMIA BLOOD		
Top fraction	5,660	39.4
Bottom fraction	187	0.1

liver biopsy. The recipient had a saturated iron binding protein, a serum iron of 250  $\mu\text{g}$  per 100 c.c., and a slightly macrocytic anemia (hematocrit 40, MCV 102). Radioactivity was expressed as counts per cubic centimeter of red cells since it was felt that the red cell mass should be relatively constant through the period of observation. This is in contrast to the other cell viability experiments in which activity is related to cubic centimeters of whole blood. There is no evident explanation for the initial lower level of radioactivity unless it is

related to the temporary red cell mass increase due to the transfusion. It is to be noticed (Fig. 3, *D*) that there is a reutilization in this patient of 25 per cent of the erythrocyte iron similar to that seen in previous animal experiments.

*E*: A third phlebotomy was performed on the donor on the twenty-first day after iron injection. This blood was preserved in acid citrate dextrose for twenty-five days at 4° to 6° C. and then transfused. The recipient, JS-FD265, was a 4-year-old child who had Cooley's anemia and had received approximately twenty transfusions before the present study. On the day of transfusion his hemoglobin was 10 grams. The survival time of these red cells and the projected survival allowing for their time in the donor and preservative are shown in Fig. 3, *E*.

*F*: Donor LK-FD244 was a patient with polycythemia vera who had been controlled by phlebotomy for two years. He showed a high-grade iron deficiency at the time of phlebotomy (MCV 70, MCH 22, MCHC 30). Six days after injection of  $\text{Fe}^{55}$ , 500 c.c. of blood were removed and transfused immediately to recipient L-FD263. The subsequent levels of circulating radio-iron per cubic centimeter of whole blood are shown in Fig. 3, *F*. The recipient in this transfusion was a 4-year-old girl who had been anemic since birth with typical findings of Cooley's anemia. She had had more than eight transfusions before the present study and a hematocrit of 18.2 prior to transfusion.

In another series of observations, normal male subjects were given  $\text{Fe}^{55}$  intravenously and beginning twenty-one days later iron was taken for four to six months by mouth immediately after meals. Subjects *F* and *R* consumed 200 Gm. of ferrous sulfate and Subject *M* 400 grams. It would appear that enough iron was absorbed in this fashion to interfere somewhat with reutilization and to allow one to approximate the life span of the erythrocyte in each case (Fig. 4). Mean cell age was calculated as 125 days (Subject *F*), 125 days (Subject *R*), and 114 days (Subject *M*).

#### DISCUSSION

The sensitivity of the counters used reach 3 per cent efficiency in the case of  $\text{Fe}^{55}$  and 20 per cent in the case of  $\text{Fe}^{59}$ . The counting levels obtained from blood samples were sufficient to allow accurate measurement of samples containing less than 5 per cent of the initial circulating radioactivity except for experiment B (Fig. 3, *B*). Here, accurate counting, that is counts more than twice background, was attained only in values showing over 30 per cent erythrocyte survival.

In the dogs and in the patient with hemochromatosis (Figs. 2 and 3, *D*) there was appreciable reutilization of the erythrocyte iron, in the neighborhood of 25 per cent. Since these new base lines are stable over a period of several weeks, it seems justifiable to assume 100 per cent destruction of transfused erythrocytes when this level has been attained. In the remaining transfusions there was no significant reutilization. Normal subjects fed iron, however, showed so great a reutilization (approximately 75 per cent) that interpretation of the data is difficult.

In these studies a population of red cells of approximately one age has been followed throughout their survival period in the circulating blood. The entrance of these cells into the circulation in the donor is shown by the utilization of the injected iron for hemoglobin production. In Fig. 5 is a composite curve of such utilization of radioiron obtained previously in normal subjects. This

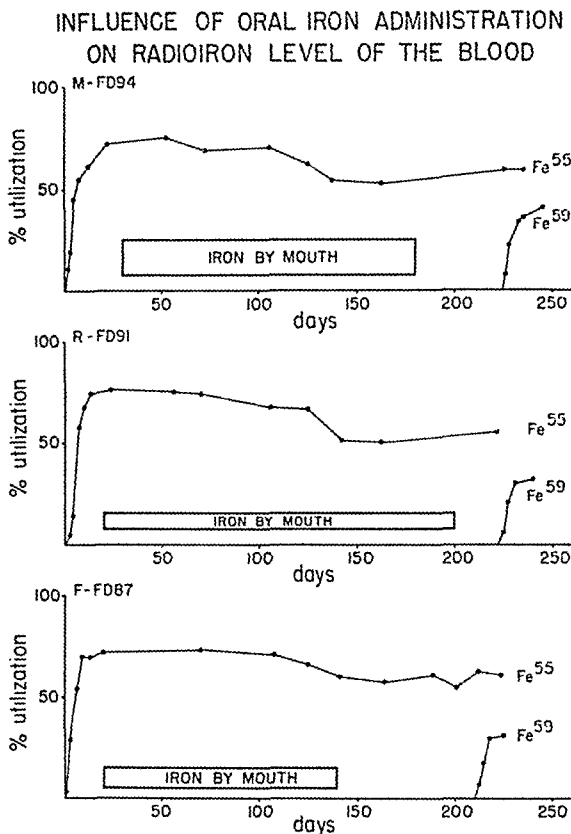


Fig. 4.—The initial utilization of radioiron in three normal subjects is shown and the subsequent circulating radioactive levels during the period of iron intake by mouth. The subsequent utilization curve with Fe<sup>59</sup> is also shown. It will be observed that between 100 and 150 days there is a significant decline in circulating radioactivity levels in each subject.

indicates that normally the fourth day will represent the mean age of tagged cells and that some 75 per cent of the total number of tagged cells appear in circulation between the second and seventh days.<sup>11</sup> In iron-deficient and pernicious anemia patients responding to treatment, the utilization may be even more rapid. For purposes of calculation in Fig. 3, corrections are made by a dotted line for the mean age of the cells, according to when they would be expected to appear in the donors circulation.

It might be questioned whether the sample taken immediately after transfusion is representative of the initial red cell level or whether during the process of transfusion which took between one-half and two hours some red cells

might already be lost from the blood stream. It has been previously demonstrated that nonviable erythrocytes may disappear very rapidly from circulation, that is, within minutes of transfusion.<sup>1, 15</sup> For this reason it appeared necessary to have some check on the initial level. Highly quantitative measurements were not possible since the blood given was only roughly measured and

### UTILIZATION OF RADIOIRON FOR HEMOGLOBIN PRODUCTION

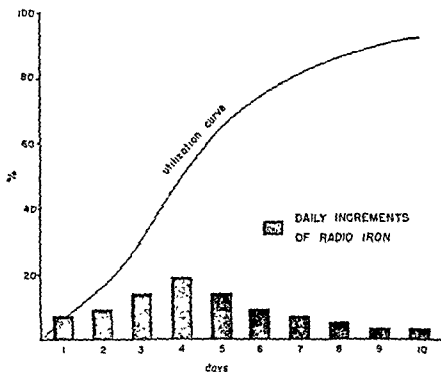


Fig. 5.—On the basis of data previously reported<sup>14</sup> a composite utilization curve is shown. This has been normalized from the 74 per cent which appears in the blood to 100 per cent so that the daily increment of radioiron incorporated in erythrocytes may be shown.

since the transfusion set was not always completely emptied of blood. In patient FW-FD129 three transfusions were given (Fig. 3, A, B, and C). The calculated blood volume on the basis of the radioactivity administered and the initial post-transfusion level were, respectively, in observations A, B, and C, 1,340 c.c., 1,400 c.c., and 1,280 cubic centimeters. Since A represents fresh normal cells which have been shown previously to be a reliable measure of the circulating mass,<sup>16</sup> the immediate survival of the other two populations of red cells may be assumed to be reasonably good. In D, the calculated radioactive cell volume was 2,160 c.c. while the Evans blue dye determination gave a cell mass of 1,975 cubic centimeters. In E, a blood volume employing T1824 immediately after transfusion gave a determination of 1,030 c.c. whereas the radioactive blood volume was 950 cubic centimeters. In patient L-FD263 (F) an Evans blue blood volume performed six months after the initial study was 1,500 c.c. as compared with the post-transfusion radioactive measurement of 1,200 c.c. in the same patient. In no instance, therefore, was there an indication of an abnormally large blood volume by radioactive measurement using the twenty minute samples. This would appear to exclude any appreciable cell destruction during the time of transfusion since such an event would result in a greatly enlarged cell volume determination by the radioactive method.<sup>15</sup>



Previous work has shown that very little radioiron injected intravenously appears in the circulating red cell mass when bone marrow synthesis is severely impaired, suggesting that only as a result of hemoglobin synthesis by the developing red cell can radioiron gain access to the erythrocyte.<sup>10, 11</sup> In the present studies, reutilization of iron is largely or completely blocked. Should there be an exchange of hemoglobin iron with plasma iron, the level of circulating activity would rapidly fall. The radioactivity in the recipients' circulation, however, followed a survival curve similar to that obtained when the pyrrol pigment<sup>17</sup> or globin of the hemoglobin molecule is isotopically tagged. It would then appear to be a safe assumption that the mature red cell throughout its life span has no appreciable exchange of iron with its surrounding environment.

In three dogs the average turnover of iron from the time of appearance in circulation to disappearance is from 95 to 109 days. In patients transfused with various populations of erythrocytes, 50 per cent of the erythrocytes had disappeared from circulation between 77 and 110 days after their appearance in circulation. The one normal cell population (Fig. 3, *A*) showed a mean cell survival of 110 days. This is consistent with other data of the life of the human red cell<sup>18</sup> and would indicate that normally there is approximately a 1 per cent daily turnover of iron through the red cell mass as a result of erythrogenesis and erythrocyte breakdown.

In contrast to differential agglutination studies in which the red cell population measured is a mixed one, in these studies red cells of a single age could be followed throughout their life period. By drawing blood shortly after injection of radioiron it was possible to obtain young cells as shown in Fig. 3, *B* and *C*. That these young cells represent reticulocytes is indicated by Table I and by other studies,<sup>19</sup> demonstrating that in fractionated blood the reticulocyte level and radioactive level show a high degree of correlation. Other studies<sup>3, 20</sup> have demonstrated that reticulocytes are capable of synthesizing heme and maturing, and that their disappearance from circulation is associated with an increase in mature red cells. The present observations would indicate that they not only mature but have a reasonably normal life span.

It is of interest that in pernicious anemia the reticulocytes after liver therapy have a normal life expectancy, since other observations on mixed population of untreated pernicious anemia macrocytes have indicated a considerably shorter life span. Iron deficiency reticulocytes as shown in Fig. 3, *C* and *F* survive a somewhat shorter than normal period, whereas the same cells in a subject with a relatively normal blood picture show a relatively normal survival. This suggests an influence of anemia per se on the life span although the varied nature of the recipients is such that no conclusions can be drawn. The different survival curves obtained with red cells from the same donor in different recipients (Fig. 3, *C* and *D*) emphasize the importance of such factors. It is of interest that storage of blood before transfusion seemed to result in an immediate loss of approximately 20 per cent of the transfused red cells while the remaining erythrocyte population survived what would appear to be a relatively normal life span.

In subjects fed iron the erythrocytes remained in their normal environment and were subjected to no manipulation. Although the great reutilization obscures the erythrocyte breakdown, the mean cell age estimated at 122 days is quite similar to the figure of 127 days obtained by Shemin and Rittenberg<sup>17</sup> using N<sup>55</sup> in the same type of study.

#### CONCLUSIONS

The reutilization of radioactive iron from broken down red cells is effectively blocked by the presence of enlarged iron stores and/or bone marrow dysfunction in animals and in human subjects. By using recipients with these characteristics, the erythrocyte unit of iron metabolism may be isolated.

Under these experimental conditions it was possible to measure the life span of a red cell population of a single age. Observations were made of tagged macrocytes, microcytes, and normal red cells in normal and anemic recipients. The reticulocytes from a patient with pernicious anemia responding to therapy and from a patient with iron deficiency have been shown to have a relatively normal life span.

The erythrocyte iron turnover measured directly is approximately 1 per cent a day in dog and in man.

There is no discernible exchange of iron between the red cell and its surroundings throughout the life span of the erythrocyte.

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## TYROSINE METABOLISM IN HUMAN SCURVY

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### INTRODUCTION

IT HAS been shown that vitamin C is intimately connected with the metabolism of tyrosine and phenylalanine. Sealock and Silberstein<sup>1</sup> reported that when tyrosine was fed to scorbutic guinea pigs, homogentisic acid was excreted, a phenomenon that could be prevented by the administration of *l*-ascorbic acid. They also demonstrated<sup>2</sup> that tyrosine, fed to guinea pigs on a diet deficient in vitamin C, resulted in the excretion of homogentisic, *p*-hydroxyphenylpyruvic, and *p*-hydroxyphenyl lactic acids, and that the administration of *l*-ascorbic acid prevented the abnormal excretion of these metabolites. Painter and Silva<sup>3</sup> fed large doses of tyrosine to scorbutic guinea pigs but found no excretion of homogentisic acid in the urine, but did observe excessive excretion of the intermediary metabolites of tyrosine. In addition, Levine and co-workers<sup>4, 5</sup> noted that premature infants fed diets of vitamin C free cows' milk containing 5 Gm. or more of protein per kilogram of body weight excreted *p*-hydroxyphenyl lactic and *p*-hydroxyphenylpyruvic acids, the excretion of which was eradicated by the administration of ascorbic acid.

The purpose of this communication is to report the effects in normal and scorbutic human beings of feeding large amounts of tyrosine and the rapid effect of *l*-ascorbic acid in correcting the abnormal tyrosine metabolism found in the individuals with scurvy.

### MATERIALS AND METHODS

The patients observed in this study were four scorbutic men aged 62, 64, 66, and 71 years, two normal men aged 22 and 29, and one normal woman aged 75.†

During the course of the studies, the scorbutic patients were maintained on a vitamin C-free diet consisting of two quarts of boiled skimmed milk, 450 grams of boiled rice, and thirty-six soda crackers daily. Sugar, coffee, and vitamin C-free purified jellies were allowed ad libitum. The two normal male subjects were allowed their usual diets. The normal female subject was maintained on the same vitamin C-free diet as the scorbutic patients, but received large supplements of crystalline ascorbic acid. Twenty-four hour samples of urine were collected daily from these patients and after suitable control periods of at least six days, all except one scorbutic patient were given 20 Gm. of powdered *l*-tyrosine§ per day orally, divided into four doses of 5 Gm. each.

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‡This 75-year-old patient was thought to have scurvy because of purpura. The purpura was of senile type only, and she was then studied as a normal patient after receiving large supplements of ascorbic acid.

§Kindly supplied by Merck & Co., Inc., Rahway, N. J.

After a period of tyrosine administration, four of the scorbutic patients received large doses of ascorbic acid in addition to the tyrosine. When possible, a control period was included after the discontinuance of tyrosine.

The ascorbic acid content of the white blood cell-platelet layer was determined by the method of Butler and Cushman.<sup>6</sup> The addition of 100 mg. of human fibrinogen (Fraction I of human plasma [Cohn]<sup>7</sup>) to 10 ml. of oxalated blood produced better separation of the white cell-platelet layer for assay. The whole blood ascorbic acid levels were determined by the method of Keuther.<sup>8</sup>

The total excretion of hydroxyphenyl compounds in the urine, expressed as tyrosine equivalents, was measured by the method of Medes.<sup>9</sup> The compounds measured by this method include tyrosine, *p*-hydroxyphenyl lactic, and *p*-hydroxyphenylpyruvic acids, and are designated "tyrosyl derivatives."

The reduction of phosphomolybdic acid by the urine as outlined by Medes<sup>9</sup> also was determined. This method, for the most part, measures the excretion of *p*-hydroxyphenylpyruvic acid, but is not specific and will react with homogentisic acid and other reducing substances. Since the reducing substances excreted by the scorbutic patients, while receiving tyrosine, were not chemically identified, their reducing power was calculated as equivalents of a standard solution of hydroquinone. The presence of homogentisic acid was tested for qualitatively by adding ferric chloride to urine that was made alkaline with sodium hydroxide. Calibration curves with hydroquinone standards for the phosphomolybdic reducing power and *l*-tyrosine for the "tyrosyl" derivatives were prepared using the Evelyn photoelectric cell colorimeter. The tyrosyl derivatives were read in the colorimeter exactly one minute after color formation to obtain maximum values. If they were read after allowing the color reaction to proceed for twenty minutes, as outlined by Levine and associates,<sup>4</sup> there was a marked drop in color intensity. The phosphomolybdic reaction was allowed to proceed for three hours before readings were made in the colorimeter.

## RESULTS

*Scorbutic Patients.—Case 1* (Fig. 1), a 62-year-old man, was admitted to the Boston City Hospital because of pain in the right thigh. He had classical signs of scurvy: a positive tourniquet test, purpura on the legs, ecchymoses of the right thigh, and hematomas around carious teeth. There was no detectable ascorbic acid present in the white blood cell-platelet layer of the blood. In a control period of six days on a vitamin C-free diet, this patient's total "tyrosyl" excretion averaged 195 mg. of tyrosine equivalents per day with a range of from 128 mg. to 248 mg. per day. In this same control period, the excretion of reducing substances as measured by the reduction of phosphomolybdic acid was equivalent to an average of 84 mg. of hydroquinone per day and ranged from 37 mg. to 146 mg. per day.

When the patient was given 20 Gm. of tyrosine daily, for six days, the excretion of "tyrosyl" compounds immediately increased, and on the sixth day of administration reached a level of 6,300 milligrams. The reducing substances of the urine remained unchanged from the control values for three days, but then steadily increased until on the sixth day of tyrosine administration the concentration was equivalent to 6,300 mg. of hydroquinone per day.

The patient was then given the daily addition of 1 Gm. of *l*-ascorbic acid orally as well as 20 Gm. of tyrosine per day for five days. The ascorbic acid was then reduced to 0.5 Gm. per day for the remainder of the study. With ascorbic acid therapy, the excretion of "tyrosyl" compounds immediately decreased and on the second day of therapy was 583 mg. and fluctuated between 453 and 1,073 mg. per day for the next seven days, when the study ended. The reducing substances of the urine showed no decrease on the first day of ascorbic acid therapy and, in fact, increased to 6,620 mg. of hydroquinone equivalents. However, on the second day of ascorbic acid therapy, the concentration of reducing substances dropped to slightly above control levels, averaged 152 mg. per day, and remained

at that value for four days. At this time the reducing power increased to a daily average of 252 mg. of hydroquinone equivalents and is interpreted as an increased excretion of ascorbic acid since this compound will reduce phosphomolybdic acid under the conditions used.

During the period of tyrosine administration, the capillary fragility increased, as measured by the tourniquet test, and there was some bleeding from the gingival hematomas. All signs of capillary fragility disappeared promptly after administration of ascorbic acid.

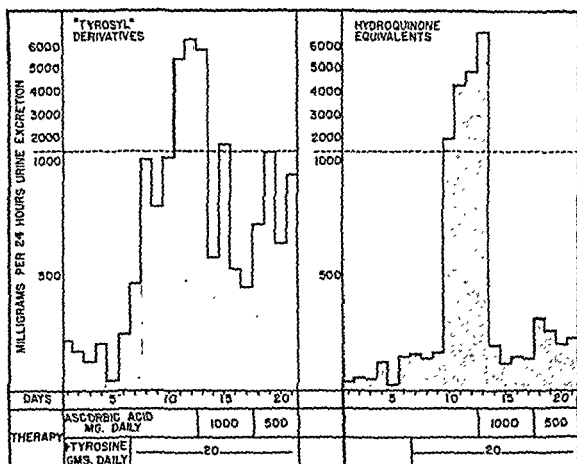


Fig. 1.—Case 1 (scurvy). Twenty-four hour excretion levels before and after treatment.

*Case 2* (Figs. 2 and 3) was a 64-year old man with the clinical picture of scurvy and with no detectable ascorbic acid in the white cell-platelet layer. He was maintained on the same vitamin C-free diet as provided for the first patient. In a nine-day control period he excreted a daily average of 109 mg. of "tyrosyl" compounds. The reducing substance of the urine in this same control period was equivalent to a daily average of 74 mg. of hydroquinone.

The patient was then given 20 Gm. of tyrosine daily orally, with a large immediate rise in excretion of "tyrosyl" derivatives. On the second day he excreted 7,700 mg., and on the fifth day, 16,120 milligrams. On the seventh day of tyrosine administration, he was given 2 Gm. of ascorbic acid orally per day. This dose was continued for five days and then decreased to 1 Gm. per day for the remainder of the observation period. Following the administration of ascorbic acid, the excretion of "tyrosyl" compounds rapidly fell and, although the patient received tyrosine for twenty-five days after institution of antiscorbutic therapy, his excretion of "tyrosyl" derivatives, after the initial drop became evident, averaged only 536 mg. per day with a daily range of from 200 to 1,400 milligrams. Tyrosine was stopped for a period of one week, during which time the average excretion dropped to 230 mg. per day. In order that the patient should act as his own control, he was given tyrosine for another eight-day period during which time the average excretion of "tyrosyl" derivatives was 312 mg. per day with the daily maximum being 400 mg. on the fourth day. In a final control period of ten days without tyrosine, he excreted an average of 140 mg. per day.

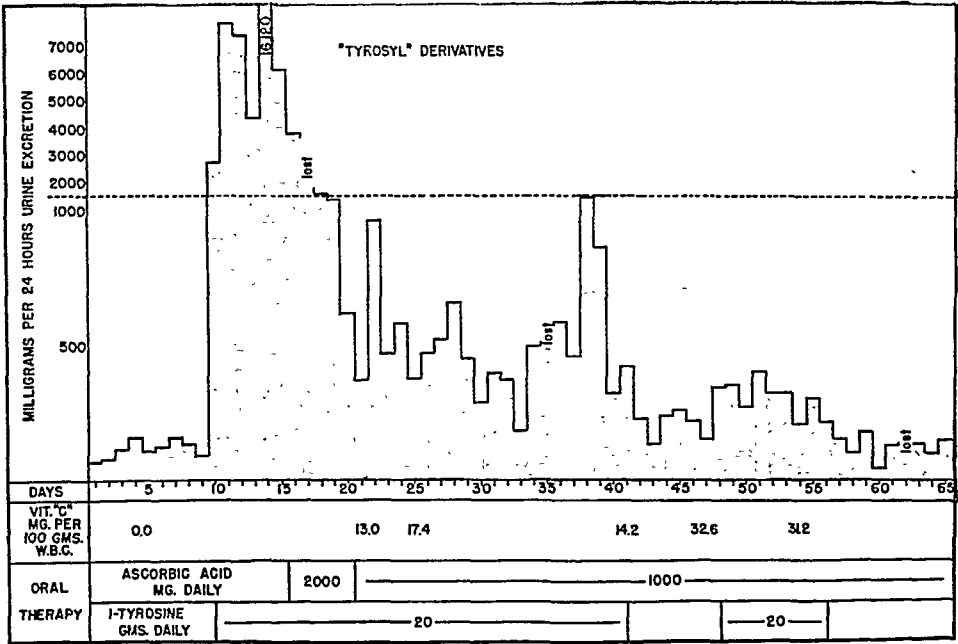


Fig. 2.—Case 2 (scurvy). Daily "tyrosyl" excretion.

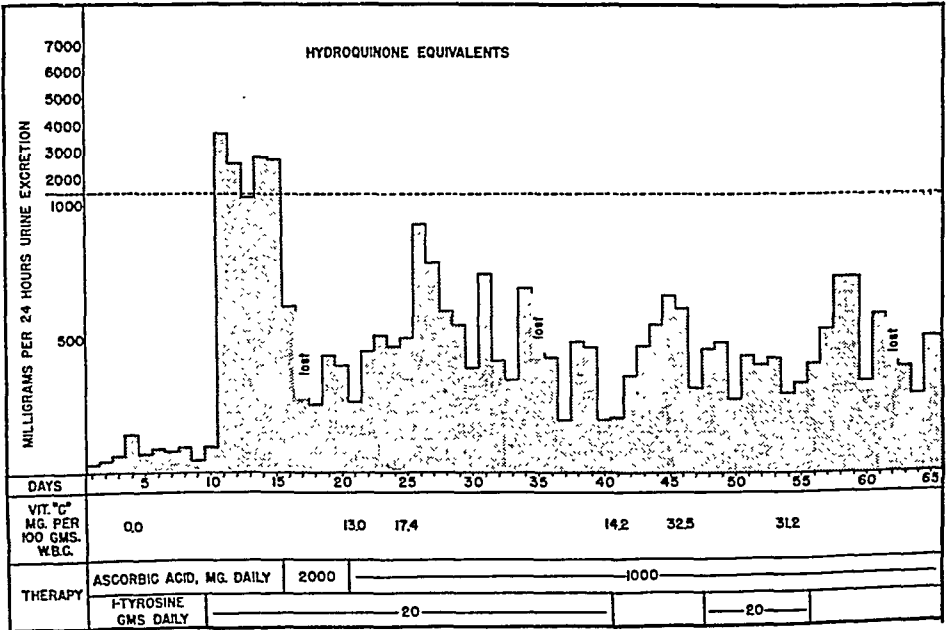


Fig. 3.—Case 2 (scurvy). Reducing substance in the urine measured as hydroquinone equivalents.

The reducing substances in this patient's urine, during the administration of tyrosine, increased rapidly to 3,480 mg. of hydroquinone equivalents on the second day and ranged above 2,500 mg. per day throughout this period except for one value of 1,340 mg. which may be in part an incomplete sample. There was a marked fall concomitant with the administration of 2 Gm. of ascorbic acid per day to an average level of 471 mg. of hydroquinone per day. Again it is believed that this higher level of excretion than in the original control period was caused by the excretion of ascorbic acid and was not due to the administration of tyrosine. This contention is borne out by the fact that when tyrosine administration was discontinued for seven days the daily average remained essentially the same (446 mg. of hydroquinone equivalents per day) and that on reinstitution of tyrosine administration the level tended to fall slightly, averaging 392 mg. of hydroquinone per day.

During the period of tyrosine administration, there was a marked increase in capillary fragility. For the first time, the tourniquet test produced numerous small hematomas over the forearm as well as petechial hemorrhages. The patient became weak and developed nausea and diarrhea; all symptoms disappeared when ascorbic acid was given.

*Case 3* (Table I) was a 71-year-old man who entered the hospital with hematomas of both calves and marked capillary fragility. There was no detectable ascorbic acid in the white blood cell-platelet layer. Throughout the study the patient was on a vitamin C-free diet and received no additional tyrosine.

In a six-day control period, before the administration of ascorbic acid, the patient excreted an average of 201 mg. of hydroxyphenyl compounds per day, and the reducing substance of the urine was equivalent to 64 mg. of hydroquinone per twenty-four hours. After this control period, the patient was given 1 Gm. of ascorbic acid a day orally for five days and then 0.5 Gm. for the remainder of the study. With ascorbic acid therapy there was no significant change in excretion of "tyrosyl" derivatives which averaged 196 mg. per day. The reducing substances in the urine remained low (averaging 92 mg. hydroquinone equivalents per day) for four days and then rose to an average excretion of 192 mg. per day which undoubtedly reflected the increased amounts of ascorbic acid being excreted in the urine.

TABLE I. CASE 3 (SCURVY): EXCRETION LEVELS BEFORE AND AFTER ADMINISTRATION OF ASCORBIC ACID

TREATMENT	DAYS	AVERAGE TYROSYL DERIVATIVES (MG./24 HR.)	AVERAGE HYDROQUINONE EQUIVALENTS (MG./24 HR.)
0	6	201	64
Ascorbic acid:			
1.0 Gm. 5 days	10	196	192
0.5 Gm. 5 days			

*Case 4* (Fig. 4). The patient entered the hospital complaining of weakness and inability to walk. He had large hematomas in the right popliteal fossa extending to the groin. There was no measurable vitamin C in the white blood cell-platelet layer of the blood. Throughout the observations the patient was maintained on the vitamin C-free diet. During a five-day control period the patient excreted a daily average of 219 mg. of "tyrosyl" derivatives. The average daily excretion of reducing substances in the urine was 20 mg. hydroquinone equivalents.

The patient was then given 20 Gm. of tyrosine daily. By the third day the "tyrosyl" excretion had increased to 11,300 mg. and the reducing substances to 3,025 milligrams.

During the period of tyrosine administration, the patient developed nausea, diarrhea, singultus, and marked lassitude. The diet intake was not maintained and urine collections could not be made. Tyrosine was discontinued and the patient received 1.5 Gm. of ascorbic acid parenterally and 2.5 Gm. orally daily. Despite the large doses of ascorbic acid and parenteral dextrose solution, the patient did not respond. He died five days after the onset of ascorbic acid therapy in an episode of pulmonary edema and shock. Post-mortem examination revealed only pulmonary congestion and no hemorrhagic phenomena.



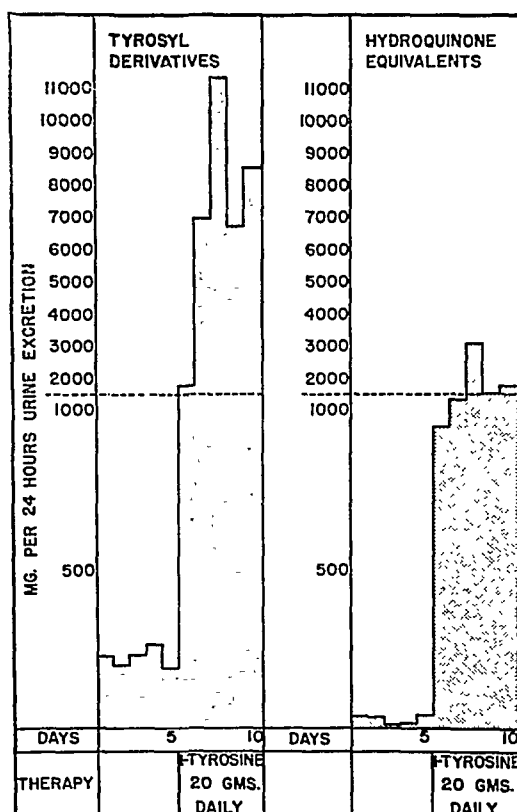


FIG. 4.—Case 4 (scurvy). Excretion levels during tyrosine administration.

TABLE II. RESPONSE OF THREE NORMAL SUBJECTS TO 20 GM. PER DAY OF L-TYROSINE ORALLY

PATIENT	L-TYROSINE (GM./DAY ORALLY)	AVERAGE TYROSYL DERIVA- TIVE (MG./24 HR.)	AVERAGE HYDRO- QUINONE EQUIVALENTS (MG./24 HR.)	DAYS IN PERIOD
Normal Subject 1	0	242	184	14
Period 1	20	755	167	11
(See Fig. 5)	0	287	135	6
Period 2*	0	328	533†	6
	20	734	588†	7
	0	290	171	5
Normal Subject 2	0	175	99	6
	20	614	112	9
	0	134	91	5
Normal Subject 3	0	218	683†	9
	20	582	887†	5
	0	230	636†	5

\*500 mg. ascorbic acid given orally during control and administration of tyrosine.

†These values are markedly elevated because of ascorbic acid administration throughout this period.

*Normal Patients.*—*Normal Subject 1* (Fig. 5) in a control period of fourteen days excreted an average of 242 mg. of "tyrosyl" compounds per day. The reducing substances in the urine in this same period were 184 mg. hydroquinone equivalents per day. He then received 20 Gm. of tyrosine daily for eleven days which resulted in a gradually increased excretion of "tyrosyl" compounds until the fifth day when it was 1,270 milligrams. Thereafter, for the next five days, a moderate fall in the excretion of "tyrosyl" derivatives occurred, followed by a second peak reaching 1,078 mg. on the last day of tyrosine administra-

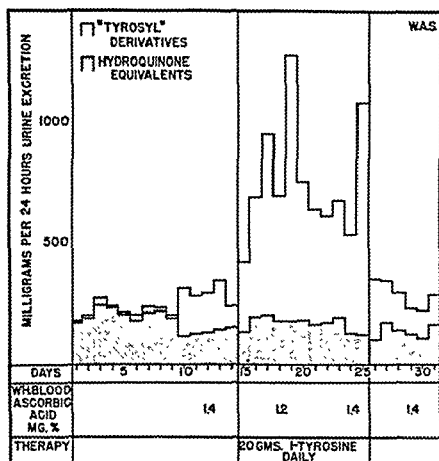


Fig. 5.—Normal Subject 1. Excretion levels of normal subject during tyrosine administration.

tion. The average excretion of "tyrosyl" derivatives during the entire period of tyrosine administration was 755 mg. per day; in a subsequent control period without tyrosine the excretion dropped to preadministration levels.

The reducing substances in this individual's urine did not change appreciably during any portion of the experiment. During the administration of tyrosine they were 167 mg. hydroquinone equivalents per day which was slightly lower than in the previous control period. Whole blood ascorbic acid levels were within normal limits and showed no significant change during the control periods or when the patient was receiving tyrosine.

In order to determine if added ascorbic acid would change the response to 20 Gm. of tyrosine per day, the same observations were repeated on this patient with the exception that he was given 500 mg. of ascorbic acid daily, starting three days before the administration of tyrosine and continuing through the entire period of tyrosine administration, Table II, Period 2. The addition of ascorbic acid in this instance did not change the response to tyrosine as evidenced by the fact that on one day the patient excreted 1,134 mg. of "tyrosyl" derivatives and the average excretion during tyrosine administration was 734 mg. per day as compared with the value of 755 mg. per day when he was not receiving ascorbic acid. The reducing substances in the urine immediately increased when he took ascorbic acid, but were not augmented further on addition of tyrosine to his diet.

*Normal Subject 2.* The response to 20 Gm. of tyrosine daily for eight days in another normal male, aged 22, is shown in Table II. It is similar to that of the previously described normal person, although this patient excreted slightly less hydroxyphenyl compounds, averaging 614 mg. per day during the period of tyrosine administration. As in the first normal subject, after an initial rise, there was a decreased excretion for three to four days followed by a second rise.

*Normal Subject 3,* a 75-year-old woman, was given the same vitamin C-free diet as described for the scorbutic patients. In addition, she received 1 Gm. of ascorbic acid orally for the first eleven days of control observation and 2 Gm. daily throughout the remainder of the study. During a control period of nine days she averaged 218 mg. per day of "tyrosyl" derivatives. She was then given 20 Gm. of tyrosine daily for a five-day period and the "tyrosyl" excretion averaged 582 mg. per day. During another five-day control period the excretion averaged 230 mg. per day.

During a nine-day control period while receiving 2 Gm. of ascorbic acid daily, the patient's average excretion of reducing substances was 683 mg. hydroquinone equivalents. During the period of tyrosine administration it rose to a daily average of 887 milligrams. In the second five-day control period the daily average was 636 milligrams. Again it is believed that the high values in the control period are associated with the reducing power of ascorbic acid. This normal subject showed a rise in reducing power of the urine during tyrosine administration. Although no complete explanation is available, the large dosage of this amino acid may have materially aided in the excretion of ascorbic acid.

#### DISCUSSION

From the foregoing results, it would seem safe to assume that the relationship between ascorbic acid and the metabolism of tyrosine in the adult human being is similar to that which had previously been demonstrated in the guinea pig<sup>1,2</sup> and the premature infant.<sup>5</sup> Sealock<sup>1</sup> has reported that normal human beings on a "diet practically free of ascorbic acid" excrete significant amounts of homogentisic acid when ingesting tyrosine, which was prevented by "reasonably large doses of crystalline ascorbic acid." However, no quantitative data are presented as to the amount of tyrosine ingested, the excretion of homogentisic acid, or the amount of ascorbic acid given.

In the present observations, the ingestion of tyrosine orally by scorbutic patients resulted in a markedly increased excretion of "tyrosyl" derivatives. The method used for the detection of the "tyrosyl" compounds theoretically measures tyrosine, *p*-hydroxyphenylpyruvic acid, and *p*-hydroxyphenyl lactic acid. As the urines were not fractionated or the compounds isolated, it is impossible to say which ones were proportionally responsible for the increased excretion of hydroxyphenyl compounds. Evidence that a large proportion was due to *p*-hydroxyphenylpyruvic acid is found in the marked increase of the reducing power of the urine. However, this reaction is not specific since another intermediary of tyrosine metabolism, namely homogentisic acid, will cause the reduction of phosphomolybdic acid. Although qualitative tests for homogentisic acid in these urines were negative, it conceivably could have been present and could have affected the quantitative reduction of phosphomolybdic acid, but could not be detected qualitatively. The absence of homogentisic acid is more in keeping with the animal studies of Painter and Silva.<sup>3</sup>

In the scorbutic patients, the increased power of the urine to reduce phosphomolybdic acid lagged behind the increased excretion of "tyrosyl" derivatives, the latter increasing immediately after the ingestion of tyrosine. Likewise, the response of the reducing substances to ascorbic acid in one patient (Case 1) was slower than that of the total hydroxyphenyl compounds. Despite the nonspecificity of the methods used, the changes noted in the scorbutic patients would seem to be the result of the tyrosine and ascorbic acid administration since the regimes were constant except for these two variables.

The excretion of "tyrosyl" derivatives by scorbutic patients who were maintained on a vitamin C-free diet did not differ appreciably from that of normal subjects when they were not receiving tyrosine. The metabolic abnormality was noted only when they were given exogenous tyrosine. Furthermore, the scorbutic patient (Case 3) who received only ascorbic acid and no tyrosine showed no statistically significant change in excretion of "tyrosyl" derivatives after the administration of ascorbic acid. Normal Subject 3 demonstrated that the dietary regime had no significant role in "tyrosyl" excretion.

The scorbutic patients demonstrated a progressive increase in capillary fragility while receiving tyrosine. While the death of the patient (Case 4) cannot be ascribed to a hemorrhagic lesion as a result of scurvy, nevertheless, all the vitamin C-deficient patients showed progression of the scorbutic skin lesions associated with increased weakness and lassitude during administration of L-tyrosine. Presumably the administration of amino acid in these large amounts placed an increased demand on the unmeasured body stores of vitamin C. In the original work of Sealock,<sup>1</sup> it is implied that the feeding of tyrosine results in a depletion or excess utilization of the ascorbic acid of the body. This phenomenon was apparently demonstrated clinically under the conditions used in the present study by the progressive intensity of the signs and symptoms of scurvy. However, in the normal individual, there was no significant decrease in the ascorbic acid content of the whole blood during the administration of tyrosine, and when these same individuals were given added ascorbic acid before and during administration of tyrosine, it did not change their response to this amino acid.

That the abnormality of the metabolism of hydroxyphenyl compounds has wider implications than in scurvy alone has support in the effect that liver extract and pteroylglutamic acid have on the metabolism of hydroxyphenyl compounds. Swendsen and co-workers<sup>10, 11</sup> have reported that patients with pernicious anemia excrete abnormally large amounts of hydroxyphenyl compounds and that the excretion of these compounds decreases to normal levels when liver extract is given. Sealock and Lepow<sup>12</sup> also have shown that antipernicious anemia extracts partially improved the tyrosine metabolism of scorbutic guinea pigs. Likewise, pteroylglutamic acid has been shown to have an effect on the metabolism of hydroxyphenyl compounds in scorbutic guinea pigs both in vivo<sup>13</sup> and in vitro experiments.<sup>14</sup> In addition, the administration of ascorbic acid to animals deficient in pteroylglutamic acid has altered the course of this deficiency.<sup>15</sup>

## SUMMARY

The metabolism of tyrosine was studied in four patients with scurvy and in three normal individuals. In six of these individuals, 20 Gm. of tyrosine per day was given by mouth for periods varying from five to thirty-one days.

The patients with scurvy, while on a vitamin C-free diet and oral administration of tyrosine, excreted large amounts of "tyrosyl" derivatives, and the ability of their urine to reduce phosphomolybdic acid increased markedly, presumably due in part to the presence of *p*-hydroxyphenylpyruvic acid. The addition of ascorbic acid to this regime in the scorbutic patients resulted in a rapid decrease in the excretion of "tyrosyl" derivatives and the disappearance of the abnormal reducing material in the urine.

The excretion of "tyrosyl" derivatives in normal individuals on tyrosine was comparable with that of scorbutic subjects during the period when they were receiving ascorbic acid. At no time did the reducing substance of the urine of normal individuals rise significantly while they were receiving tyrosine. The addition of ascorbic acid to a normal individual's diet did not change the response to the administration of tyrosine.

It is concluded that individuals with scurvy have a marked defect in the metabolism of tyrosine and hydroxyphenyl compounds and that previous experiments with scorbutic animals and premature infants are in general agreement with the findings reported here.

It seems unlikely that this defective metabolism of tyrosine plays a significant role in the clinical picture of scurvy since the excretion of "tyrosyl" derivatives was not remarkable unless added tyrosine was given.

The clinical status of scurvy is made more severe during tyrosine therapy with increase in capillary fragility. These manifestations are completely controlled when ascorbic acid is added to the diet despite continued administration of tyrosine.

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# OBSERVATIONS ON THE ETIOLOGIC RELATIONSHIP OF ACHYLIA GASTRICA TO PERNICIOUS ANEMIA

## XI. HEMATOPOIETIC ACTIVITY IN PERNICIOUS ANEMIA OF A BEEF MUSCLE EXTRACT CONTAINING FOOD (EXTRINSIC) FACTOR UPON INTRAVENOUS INJECTION WITHOUT CONTACT WITH GASTRIC (INTRINSIC) FACTOR

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PREVIOUS observations<sup>1, 2</sup> have shown that 200 Gm. of beef muscle are hematopoietically active in addisonian pernicious anemia when 150 ml. of normal human gastric juice also are given by mouth within six hours, or preferably simultaneously, as a daily regime for a period of ten days. If an acid mixture of beef muscle and gastric juice is incubated for twelve hours for the purpose of effecting peptic digestion of the beef muscle and is then neutralized, it also is active.<sup>3</sup> On the other hand, if the incubated mixture is heated to 100° C. for five minutes, its hematopoietic activity as determined by oral administration is destroyed,<sup>3</sup> whereas that of whole liver or liver extract is not detectably affected by such a procedure. From this it was assumed that the thermostable antipernicious anemia principle of liver was *not* formed by the incubation procedure in vitro and that the effect of heat was merely to destroy a thermolabile factor in the gastric juice.

If a beef muscle and gastric juice mixture is given at an acid pH (1.8 to 5) subsequent to the twelve-hour acid incubation period, no hematopoietic effect appears.<sup>3</sup> If, however, this acid incubated mixture is treated with alkali to give a pH of 5 to 7 just prior to oral administration, it is active. This suggested that a preliminary chemical interaction occurred between the so-called extrinsic factor of beef muscle and the intrinsic factor of normal human gastric juice at or about neutrality within the intestinal tract. Such a reaction was conceived as necessary for the eventual formation of the antipernicious anemia principle in the body. It seemed probable, therefore, that the extrinsic factor was chemically different from the antipernicious anemia principle of liver.

Because of this evidence, the increased hematopoietic activity in pernicious anemia of liver<sup>4</sup> and of relatively crude liver extracts<sup>5</sup> when given orally together with normal human gastric juice was originally assumed to indicate the presence of extrinsic factor as well as of the antipernicious anemia principle.<sup>6</sup> However, it was later shown<sup>7</sup> that even refined liver extracts when given by

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mouth were potentiated in their hematopoietic effect by gastric juice and that destruction of the antipernicious anemia principle of such extracts by hydrolysis with 5 per cent sulfuric acid resulted likewise in the loss of extrinsic factor activity. Recently it was found<sup>7</sup> that the hematopoietic activity of orally administered pure vitamin B<sub>12</sub> derived from liver (presumably the antipernicious anemia principle) also was enhanced by the simultaneous administration of normal human gastric juice—but that the activity of the combination when given orally was not so great as that of the vitamin B<sub>12</sub> when administered alone parenterally.

The observations reported here concern questions obviously raised by these findings: (1) Can extrinsic factor *alone* (beef muscle) act directly as the antipernicious anemia principle; that is, is a suitable preparation of beef muscle, like vitamin B<sub>12</sub>, hematopoietically effective in pernicious anemia upon parenteral administration without contact with gastric juice? (2) Is the hematopoietic potentiating action of intrinsic factor in pernicious anemia *specific* only for vitamin B<sub>12</sub> and chemically similar substances in beef muscle and in other foods; or does intrinsic factor also facilitate the nonspecific absorption or otherwise enhance the hematopoietic action of other substances?

#### METHODS

All patients studied had Addisonian pernicious anemia either in relapse or without previous treatment. The diagnosis was established by the characteristic blood and bone marrow morphology and by the presence of histamine-fast achlorhydria. It was confirmed in each patient by one or more reticulocyte responses to specific therapy and eventually by a return to normal blood values as a result of liver extract therapy. During the observations the basal diet of the patients contained no meat, fish, or eggs, and little milk. The administration of the test substances, including the normal human gastric juice, was made at 8 P.M. The last meal of the day, consisting of tea and white toast, was at 4 P.M. This separated the test substance by at least four hours from any food constituent considered likely to contain extrinsic factor.

The attempted plan of the observations was to administer to the patients during three consecutive periods of ten or more days each a uniform daily dose of extrinsic factor derived from 400 Gm. of beef muscle first orally, then orally with 150 ml. of normal human gastric juice, and finally intravenously *without* gastric juice. In evaluating the hematopoietic activity of the various preparations used, the method of serial reticulocyte responses<sup>8</sup> was employed.

Formijne<sup>9</sup> has shown that a 70 per cent alcohol extract of beef muscle possesses extrinsic factor activity in pernicious anemia. Consequently the preparation of such an extract was undertaken, starting with a concentrated aqueous extract of lean beef muscle,\* of which 1 Gm. was derived from 14 to 16 Gm. of beef muscle. Following dilution of the aqueous extract with an equal volume of distilled water, a 70 per cent alcohol concentration by volume was made by the addition of suitable amounts of 95 per cent ethyl alcohol. The

\*Kindly supplied by Dr. William Peabody of the Valentine Company, Inc., Richmond, Va.



TABLE I. EFFECT OF DAILY

CASE PERIOD DAY OF THERAPY	10 ML. MEAT EXTRACT* ORALLY						10 ML. MEAT EXTRACT* AND 150 ML. GASTRIC JUICE ORALLY							
	109		110		111		105		109		110		111	
	I		I		I		I		II		II		II	
	R.B.C. (MIL.)	RET. (%)	R.B.C. (MIL.)	RET. (%)	R.B.C. (MIL.)	RET. (%)	R.B.C. (MIL.)	RET. (%)	R.B.C. (MIL.)	RET. (%)	R.B.C. (MIL.)	RET. (%)	R.B.C. (MIL.)	RET. (%)
0	1.28	0.4	1.72	1.3	1.82	0.7	1.87	1.5	1.66	0.8		1.9		1.0
1		0.4		0.7		0.9		1.3		0.6		1.3		1.4
2		0.6		1.0	1.75	0.7	1.83	1.3		0.3		1.5		1.9
3		0.3	1.73	0.9		1.3		1.4	1.62	0.5	2.15	1.6	1.42	1.5
4	1.28	0.6		1.1		1.4	1.78	1.2		1.4		1.1		1.9
5	Tsf.	0.7		1.4	1.54	1.2		1.3		1.3		1.3		2.6
6		0.7	1.96	1.9		0.8	1.91	2.3	1.35	1.4	1.85	2.0	1.40	2.5
7	1.59	0.6		3.4		0.9		3.8		1.0		2.5		3.2
8		0.7		2.1	1.58	0.8	1.90	5.2		1.2		1.7		3.7
9		1.2	2.24	2.6		1.0		5.9	1.59	1.6	2.53	1.7	1.52	4.5
10	1.66	0.8		1.9	Cont'd 111-II		2.11	6.8		2.9		1.7		4.1
11								7.2		2.3		1.5		4.8
12	Cont'd 109-II		Cont'd 110-II				2.49	3.7	1.50	1.5	Cont'd 110-III		1.54	
13								5.3		1.8				
14							2.58	2.3		2.3				2.2
15							End		1.40	3.3			Cont'd 111-III	
16										5.0				
17										8.0				
18									1.44	7.2				
19										7.4				
20										10.9				
21									1.99	7.9				
22										8.3				
23										9.1				
24									2.10	10.1				
25										7.1				
26										3.1				
									Cont'd 109-III					

Tsf., Transfusion of 250 ml. of packed red blood cells.

\*10 ml. of meat extract are derived from 400 Gm. of lean beef muscle.

†Rule indicates end of therapy in period.

flocculent precipitate resulting was allowed to settle overnight at 5° C. The supernatant liquid was then filtered through a fluted paper filter and the precipitate washed on the paper with one-fifth the initial volume of 70 per cent alcohol. The precipitate was then discarded. Thereafter, the alcoholic filtrate was concentrated by free evaporation on electric hot plates. At the final point of concentration, the boiling point reached 108° ± 4° C. The volume of the concentrated filtrate was then so adjusted with distilled water that 1 ml. was derived from 40 Gm. of the original beef muscle. The filtrate was then bottled and autoclaved for twenty minutes. Throughout the preparation of the alcoholic extract, precautions were maintained to employ pyrogen-free fluids and apparatus.

When the preparation was given orally, 10 ml. were diluted in either 150 ml. of physiologic saline or in 150 ml. of neutralized normal human gastric juice secreted after the injection of histamine. When given intravenously, 10 ml. of the extract were mixed with 500 ml. of 5 per cent sterile dextrose and water. The slow intravenous administration of the meat extract was followed in a few instances by moderate or mild febrile reactions. These reactions

## ADMINISTRATION OF VARIOUS SUBSTANCES

10 ML. MEAT EXTRACT* INTRAVENOUSLY										1 µG VITAMIN B <sub>12</sub> INTRAMUSCULARLY					
106		107		108		109		110		111		108		111	
I		I		I		III		III		III		II		IV	
R.B.C. (MIL.)	RET. (%)	R.B.C. (MIL.)	RET. (%)	R.B.C. (MIL.)	RET. (%)	R.B.C. (MIL.)	RET. (%)	R.B.C. (MIL.)	RET. (%)	(MIL.) R.B.C.	RET. (%)	R.B.C. (MIL.)	RET. (%)	R.B.C. (MIL.)	RET. (%)
2.18	0.2	2.07	0.9	1.79	0.3	2.16	2.0		1.5		2.2	1.55	5.6	1.79	1.2
	0.4		0.8		0.2		4.2	2.32	2.1		1.7		5.0		1.8
2.12	0.0		0.9	1.51	0.3		5.0		—	1.41	1.6		4.3		2.8
	0.0		0.8		0.2	2.15	2.9		1.3		1.2	1.67	3.1	2.23	3.2
2.39	0.1		1.2		0.4		1.2	2.39	1.6		2.2		3.2		4.1
	0.4	2.17	2.0	1.44	0.6		1.8		2.5		2.6		4.2		4.9
2.21	1.8		2.9		0.4	2.30	2.5		3.3	1.51	4.6	1.80	5.1	2.48	7.6
	4.2		3.1		0.6		4.4	2.58	3.9		5.5		6.3		4.6
2.23	7.3		4.9		0.8		6.4		3.7		6.7		7.3		5.6
	8.3		4.6	1.51	1.3	2.63	4.8		2.6	1.60	5.9	2.00	5.3	2.58	4.8
2.49	9.3		3.6		2.1		5.4		1.1		5.6		4.8		4.7
	6.4		2.5		2.8		2.7		End		7.8		3.9		3.9
2.25	3.3	2.69	3.1		3.6	3.23	1.2			1.61	7.8		5.8	2.48	3.7
	2.6		3.8		4.7	End					6.3	2.13	3.6		2.0
2.58	2.6		2.4	1.39	5.4						7.3		5.3	End	
End			1.8		5.9						1.63		5.3		
		3.01	1.3		5.7						6.0	2.31	5.2		
			1.6	1.55	5.6						5.4	End			
			1.6	Cont'd				0.5 µg of vitamin B <sub>12</sub> daily		1.75	4.2				
		3.09	2.0	108-II						1.74	3.9				
		End									2.1				
											1.4				
										1.79	1.2				
										Cont'd 111-IV					

usually developed with the first two or three injections and did not thereafter recur. No signs of subsequent sensitization to the extract or to beef appeared in any of the patients.

## OBSERVATIONS

*Is Extrinsic Factor Hematopoietically Effective Upon Parenteral Administration Without Intrinsic Factor?* The results of the observations upon the hematopoietic effect of the 70 per cent alcohol extract of beef muscle in seven patients with pernicious anemia are shown in Table I. Although the individual reticulocyte peaks are small, the trend of their values and the time of their maxima and of the clinical improvement exhibited by the patients convinced the observers of the validity of the conclusions drawn under the usual carefully controlled conditions of the tests. In one patient, Case 105, the extract derived from 600 Gm. of beef muscle given orally daily together with 150 ml. of normal human gastric juice was hematopoietically active. In two patients, Cases 109 and 111, the extract derived from 400 Gm. of beef muscle was inert upon daily oral administration, was slightly active upon daily oral administration with 150 ml. of normal human gastric juice, and was still more active upon daily intravenous administration without gastric juice. In one patient, Case 110, the preparation of extrinsic factor was weakly active upon

daily oral administration, was slightly more active when given with normal human gastric juice, and was still more active upon intravenous administration without gastric juice. Three patients, Cases 106, 107, and 108, received the extract without gastric juice by intravenous injection only. A definite reticulocyte response appeared in each instance. In Case 107 the red blood cell count

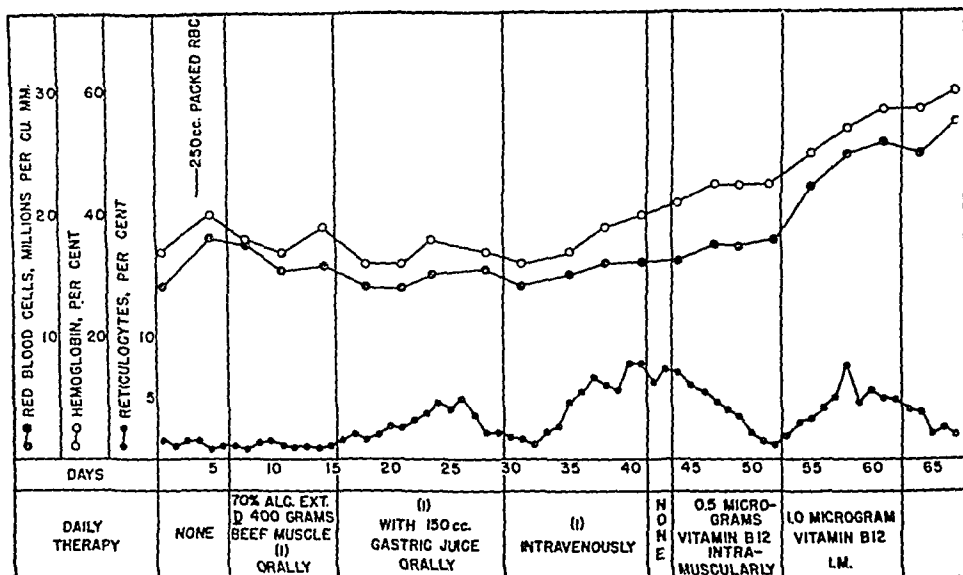


Fig. 1.—Hematopoietic effects in Case 111 of the uniform daily administration of various substances in successive periods as shown. (I) refers to 10 ml. of 70 per cent alcohol soluble meat extract derived from 400 Gm. of beef muscle. Note the three successive reticulocyte peaks indicating, respectively, activation of the extract upon oral administration with normal human gastric juice, its greater activity upon intravenous administration without gastric juice, and finally the still greater activity of vitamin B<sub>12</sub> when given in a dose of 1  $\mu$ g daily.

increased in twenty days from 2.07 to 3.09 millions per cubic millimeter. In two patients, Cases 108 and 111, the daily intramuscular administration of 1  $\mu$ g of crystalline vitamin B<sub>12</sub>\* following the period of intravenous administration of the beef muscle extract resulted in another definite reticulocyte peak. From this it was inferred that the hematopoietic activity of the extrinsic factor preparation upon parenteral administration was distinctly less than that of 1  $\mu$ g of vitamin B<sub>12</sub>. The results in Case 111 are shown in Fig. 1.

*Is the Hematopoietic Potentiating Action of Intrinsic Factor Specific?*  
In order to determine whether or not normal human gastric juice possesses a nonspecific effect in enhancing absorption from the alimentary tract in pernicious anemia, the blood levels of test substances presumably unrelated to vitamin B<sub>12</sub> were followed after oral administration to seven patients with pernicious anemia. Most of the observations were made before treatment, but a few were conducted subsequently during remission after specific therapy. Thus, blood glucose and blood tyrosine levels were determined following the

\*Kindly supplied by Dr. Augustus Gibson of Merck & Co., Inc., Rahway, N. J.

oral administration of single doses of 50 and 10 Gm. respectively of those substances to fasting patients. The test substances were mixed together and given simultaneously with either 150 ml. of normal human gastric juice or with an equal amount of physiologic salt solution upon alternate days. Blood alpha-amino nitrogen and nonprotein nitrogen levels were similarly followed after the ingestion of 50 Gm. of vitamin-free casein (Labeo) (previously shown to contain no extrinsic factor)<sup>10</sup> with either physiologic saline or normal human gastric juice upon different days. Glucose determinations were made according to the method of Folin and Wu<sup>11</sup> and of Folin,<sup>12</sup> tyrosine by the method of Bernhart and Schneider,<sup>13</sup> alpha-amino nitrogen by the method of Krauel,<sup>14</sup> and nonprotein nitrogen by the method of Folin and Wu.<sup>15</sup> As shown in Table II, no consistently enhanced absorptive effects upon glucose, L-tyrosine, or casein were observed that could be attributed to the gastric juice in any of the patients with pernicious anemia so tested, before effective therapy. Not shown are the similar negative results obtained in a few patients after treatment.

The possible hematopoietic potentiating effect of normal human gastric juice upon synthetic pteroylglutamic acid,\* either by increasing its absorption or by other action, was also assayed in two patients, Cases 112 and 113, not included in the tables. To each, 0.5 mg. of pteroylglutamic acid was given orally daily during a first period of ten or more days together with 150 ml. of physiologic saline, and during a second period of ten days the same amount of pteroylglutamic acid was given orally daily together with 150 ml. of normal human gastric juice. In the first period of Case 112 a reticulocyte peak of 9.3 per cent was reached on the eighth day following an initial red blood cell count of 1.63 million per cubic millimeter. In Case 113 the corresponding value was 8.1 per cent reticulocytes on the eleventh day following an initial red blood cell level of 1.86 million per cubic millimeter. When gastric juice replaced the physiologic saline in the second periods no detectable reticulocyte peaks appeared in either patient. Thus, no hematopoietic potentiating effect of normal human gastric juice upon pteroylglutamic acid was observed.

#### DISCUSSION

In 1938 Dock<sup>16</sup> wrote, "It seems probable that beef muscle contains the same material as liver, but in quantities too small to be effective unless absorption is increased by gastric or enteric ferment. For some reason, parenteral administration of muscle extract seems not to have been tried, but extract of muscle which had been incubated with gastric juice was potent in Wilkinson's experiments."

The potentiation of the hematopoietic activity of vitamin B<sub>12</sub> with gastric juice upon oral administration<sup>7, 17</sup> and the present confirmation of our preliminary observation<sup>7</sup> that the extrinsic factor of beef muscle is hematopoietically active upon parenteral administration *without gastric juice* go far to support Dock's surmise. It is of interest that Wilkinson<sup>18</sup> employed by daily intramuscular injection an extract derived from about the same amount of

\*Folvite, kindly supplied by Dr. Guy W. Clark of Lederle Laboratories, Inc., Pearl River, N. Y.

TABLE II. NEGATIVE EFFECT OF NORMAL HUMAN GASTRIC JUICE IN AUGMENTING THE ABSORPTION OF ORALLY ADMINISTERED GLUCOSE, L-TYROSINE, OR DIGESTION PRODUCTS OF VITAMIN-FREE CASEIN

CASE	DATE	FOLLOWING 50 GM. OF GLUCOSE BY MOUTH					VEHICLE (150 ML.)	FOLLOWING 10 GM. OF L-TYROSINE BY MOUTH				
		BLOOD GLUCOSE (MG. PER 100 ML.)						BLOOD TYROSINE (MG. PER 100 ML.)				
		FASTING	1 HR.	2 HR.	3 HR.	4 HR.		FASTING	1 HR.	2 HR.	3 HR.	4 HR.
107	2/7/49	87	163	174	117	89	Saline*	1.2	5.8	8.6	11.2	11.8
	2/8/49	117	248	271	186	125	N.H.G.J.	1.1	4.2	6.1	5.1	6.7
	2/9/49	77	170	167	117	78	Saline					
	2/11/49	86	191	164	96	106	N.H.G.J.	1.6	8.1	9.6	8.7	8.5
108	12/15/48	131	133	140	131	120	Saline	1.3	3.2	3.5	4.5	4.2
	12/16/48	110	134	135	114	92	N.H.G.J.	2.1	4.7	6.9	7.4	5.0
	12/17/48	101	135	132	117	101	Saline	2.5	4.3	4.6	6.1	5.9
	12/20/48	125	118	143	144	134	N.H.G.J.	1.6	3.4	-	4.6	7.1
109	3/1/49	85	-	88	107	87	Saline	1.4	2.1	2.6	3.5	4.5
	3/3/49	101	102	133	111	87	N.H.G.J.	1.4	2.6	3.8	4.3	5.3
110	3/1/49	64	85	86	66	63	Saline	1.2	2.9	4.4	4.6	4.5
	3/3/49	96	106	116	94	79	N.H.G.J.	1.5	2.9	3.9	4.7	4.7
111	2/2/49	114	138	143	133	136	Saline	1.1	3.0	4.4	5.2	4.2
	2/5/49	96	144	138	113	90	N.H.G.J.	1.4	3.0	4.0	4.1	3.2
	2/11/49	107	101	113	81	93	N.H.G.J.	1.5	4.4	6.5	6.4	4.1
	3/23/49	80	150	122	92	86	Saline	1.2	2.7	3.4	3.3	4.3
112	3/28/49	-	88	145	126	75	N.H.G.J.	1.1	3.2	4.0	6.6	6.8
CASE	DATE	FOLLOWING 50 GM. OF VITAMIN-FREE CASEIN BY MOUTH					VEHICLE (150 ML.)	BLOOD ALPHA-AMINO NITROGEN (MG. PER 100 ML.)				
		BLOOD NONPROTEIN NITROGEN (MG. PER 100 ML.)										
		FASTING	1 HR.	2 HR.	3 HR.	4 HR.		FASTING	1 HR.	2 HR.	3 HR.	4 HR.
109	2/26/49	27.6	32.0	33.6	32.8	35.2	Saline	5.7	6.6	7.7	8.1	7.6
	2/25/49	26.8	24.4	34.4	33.2	37.2	N.H.G.J.	6.7	6.7	7.4	7.7	8.0

\*0.85 per cent solution of NaCl.

†Normal human gastric juice.

beef muscle as we used. Although it was administered subsequent to incubation with an extract of hog mucosa, it seems probable that this was the first demonstration, though not then so interpreted, of the hematopoietic activity of a beef muscle extract upon parenteral administration.

The potentiating effect of normal human gastric juice upon orally administered vitamin B<sub>12</sub> has been demonstrated.<sup>7, 17</sup> The present observations fail to show any enhanced absorption by gastric juice of *L*-tyrosine, glucose, or of the digestive products of orally administered casein. Pteroylglutamic acid was not demonstrably more active in producing a reticulocyte response when given with gastric juice than with an equal amount of physiologic salt solution. On the basis of the present evidence, then, the action of intrinsic factor appears to be a specific one—presumably a hematopoietic potentiating effect on substances possessing identity with or chemical similarity to vitamin B<sub>12</sub>.

There is suggestive evidence, however, that vitamin B<sub>12</sub> may exist in nature, not necessarily in the pure form employed in these observations, but rather as protein "conjugates" or analogues. Thus, Smith and Parker<sup>18</sup> noted the "dramatic" increase in concentration effected by hydrolysis with trypsin of liver fractions containing vitamin B<sub>12</sub>. According to Cohn and associates,<sup>20</sup> the antipernicious anemia activity of liver may remain largely with the proteins if their denaturation is avoided during the chemical fractionation of the liver. A synthetic modification of vitamin B<sub>12</sub> known as vitamin B<sub>12a</sub><sup>21</sup> with definite though reduced hematopoietic activity has recently been made by hydrogenation of vitamin B<sub>12</sub>. By chromatography a second crystalline variant, designated as vitamin B<sub>12b</sub>, has been prepared from liver and appears to possess biologic activity in both chick and microbial assays.<sup>22</sup>

Finally, from the comparative degrees of potentiation of pure vitamin B<sub>12</sub> and of the present beef muscle extract by normal human gastric juice, at least an impression of a discrepancy is conveyed. Thus, in our previously reported observations,<sup>7</sup> with the exception of Case 102, the potentiation of the activity of 5 µg of pure vitamin B<sub>12</sub> by gastric juice, though definite, was not remarkable in terms of reticulocyte responses. Yet in the present observations upon Cases 108 and 111, the daily dose, 10 ml., of the beef muscle extract employed, although possessing upon intravenous injection distinctly less than the hematopoietic activity of 1 µg of pure vitamin B<sub>12</sub>, was nevertheless capable of detectable increase in hematopoietic effect when given orally in the same dosage with gastric juice. According to microbial assays using the organism *Lactobacillus lactis* Dorner,\* the vitamin B<sub>12</sub> content of 10 ml. of this beef muscle extract was 0.9 microgram. Assay of the same extract using the organism *Lactobacillus leichmannii*<sup>23</sup>† demonstrated 0.37 µg of vitamin B<sub>12</sub> per 10 milliliters. In the latter method the effect of bacterial growth promoting substances other than vitamin B<sub>12</sub> is stated to be determined by difference following destruction of the vitamin B<sub>12</sub> in an aliquot portion of the sample by alkaline hydrolysis. This procedure completely destroyed the microbial activity for

\*These assays were kindly performed by the laboratories of Merck & Co., Inc., Rahway, N. J.

†These assays were kindly performed by Dr. Thomas H. Jukes of Lederle Laboratories, Inc., Pearl River, N. Y.

*L. leichmannii*, and so presumably indicates the absence of significant quantities of thymidine or of other desoxyribosides. The results obtained upon microbial assay are seemingly in reasonably close agreement with each other and with the clinical effects upon intravenous injection in Cases 108 and 111. However, as indicated, the potentiating effect of gastric juice upon the beef muscle extract when orally administered is perhaps greater than would be expected from less than 1  $\mu\text{g}$  of vitamin  $\text{B}_{12}$ . Thus, it is possible that substances in the beef muscle extract other than microbially active vitamin  $\text{B}_{12}$  may be effective as food (extrinsic) factor in pernicious anemia. Yet, so far as can be inferred from the negative results of the studies with glucose, *L*-tyrosine, casein, and pteroylglutamic acid, the function of the gastric (intrinsic) factor is a specific one.

#### SUMMARY AND CONCLUSIONS

1. Observations were made during successive periods of ten or more days in seven patients with addisonian pernicious anemia employing a 70 per cent alcohol extract of beef muscle as a source of food (extrinsic) factor.

2. When 10 ml. of the extract derived from 400 Gm. of beef muscle were given daily by mouth to these patients, a detectable reticulocyte response appeared in only one instance. When the extract was given together with 150 ml. of normal human gastric juice to four patients, reticulocyte responses appeared in all instances, and when the material was subsequently given intravenously without gastric juice to three of these patients, another reticulocyte response appeared, thus indicating greater hematopoietic activity.

3. In two patients it was shown that the hematopoietic effect of 10 ml. of the beef muscle extract upon daily intravenous injection was less than that of the daily intramuscular injection of 1  $\mu\text{g}$  of crystalline vitamin  $\text{B}_{12}$ . Microbial assays indicated that 10 ml. of the beef muscle extract contained from 0.37 to 0.9  $\mu\text{g}$  of vitamin  $\text{B}_{12}$  activity.

4. However, judging from our previous observations upon the potentiation of 5  $\mu\text{g}$  of crystalline vitamin  $\text{B}_{12}$  by normal human gastric juice, the hematopoietic activity of the beef muscle extract when given orally with gastric juice appeared to be surprisingly great.

5. Only this fact suggests that substances in the meat extract other than vitamin  $\text{B}_{12}$  are susceptible of potentiation by normal human gastric juice upon oral administration in pernicious anemia.

6. No evidence was obtained for a nonspecific effect of gastric (intrinsic) factor in increasing the hematopoietic action of pteroylglutamic acid or in promoting the intestinal absorption of glucose, *L*-tyrosine, or the digestion products of casein.

We desire to express our indebtedness to the late Dr. Lionel Berk, whose death unhappily interrupted the preliminary work that led to the present observations. We have received much valuable advice from Dr. Arnold B. Welch and Dr. Robert W. Heinle of the School of Medicine of Western Reserve University. Finally, we are grateful to Miss Phyllis Gordon, Mrs. Katharine Bingham, and Miss Angela Pasquariello for the performance of the blood examinations.

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# FURTHER OBSERVATIONS ON THE USE OF THE URINARY PIGMENT-CREATININE RATIO FOR THE MEASUREMENT OF BASAL METABOLIC RATE

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IN A previous communication,\* it was found that an accurate correlation existed between the basal metabolic rates as determined by the respiratory calorimeter and the urinary pigment-creatinine ratio (P/C).† On the basis of statistically valid results obtained in 156 adult female subjects and 57 adult male subjects, regression equations were derived which permit the calculation of the basal metabolic rate from the urinary pigment-creatinine ratio. These equations are:

Male  
Female

$$\begin{aligned}\text{B.M.R.} &= 57.0 + 0.25 \text{ P/C} \\ \text{B.M.R.} &= 54.7 + 0.22 \text{ P/C}\end{aligned}$$

This present study is an analysis of the results obtained in comparing the basal metabolic rates determined by the respiratory calorimeter and the pigment-creatinine ratio in 740 observations made on 424 adult patients. Of the 740 observations, 457 (61.7 per cent) were on euthyroid patients, 168 (22.8 per cent) were on hyperthyroid patients, and 115 (15.5 per cent) were on hypothyroid patients. The clinical material consisted of hospital, private, and out-patient department patients.

## RESULTS

We have previously reported\* that the determination of the pigment-creatinine ratio is unreliable in the presence of increased bilirubinemia, azotemia, and in the presence of hemoglobinuria and drugs and foods which impart an abnormal color to the urine (e.g., riboflavin, aureomycin, beets, and rhubarb). There were 32 such cases in our series. The pigment-creatinine determination is also deficient in measuring hypometabolism, rarely indicating metabolic rates of less than -15 per cent. There were 115 observations on cases of this type in our series. Thus, there were 147 instances in which the pigment-creatinine determination would not be expected to be of diagnostic value.

Of the remaining 593 observations, the B.M.R. as determined by the respiratory calorimeter and the pigment-creatinine ratio agreed with each other and with the clinical findings in 508 cases (85.6 per cent). Of these 508 observations, 380 (64.0 per cent) were on euthyroid patients and 128 (21.6 per cent) were on hyperthyroid patients. In 58 cases (9.8 per cent) the B.M.R. as determined by the pigment-creatinine ratio was more consistent with the clinical findings than the B.M.R. determined on the basis of oxygen con-

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†The chemical determinations involved in this test can be done in about twenty-five minutes and can be performed easily in any routine chemical laboratory.

TABLE I. RESULTS OF TESTS—EXCLUDING OBSERVATIONS IN WHICH P/C WOULD NOT BE USED FOR DIAGNOSTIC PURPOSES\*

RESULT	NUMBER OF OBSERVATIONS	PER CENT OF TOTAL
B.M.R. and P/C agree with each other and with the clinical findings		
a. Euthyroids	380	64.0
b. Hyperthyroids	128	21.6
	508	85.6
P/C more accurate than B.M.R.		
a. Euthyroids	48	8.1
b. Hyperthyroids	10	1.7
	58	9.8
B.M.R. more accurate than P/C		
a. Euthyroids	14	2.4
b. Hyperthyroids	13	2.2
	27	4.6
Total	593	100.0

\*The P/C determination is not reliable in cases of jaundice, azotemia, and in the presence of hemoglobinuria or drugs that color the urine (32 cases); or in hypothyroidism—metabolism below -15 per cent (115 cases).

sumption. Of these 58 cases, 48 (8.1 per cent) were euthyroid patients and 10 (1.7 per cent) were hyperthyroid patients. In 27 cases (4.6 per cent) the B.M.R. determined by the pigment-creatinine ratio was less in agreement with the clinical findings than was the B.M.R. as determined by the respiratory calorimeter. Of these 27 cases, 14 (2.4 per cent) were euthyroid patients and 13 (2.2 per cent) were hyperthyroid patients. Thus, in 566 observations (95.4 per cent), the B.M.R. as determined by the pigment-creatinine ratio in normal and hyperthyroid patients was of diagnostic value. In the remaining 27 cases (4.6 per cent) the pigment-creatinine metabolic rate was inaccurate.

#### DISCUSSION

The observation that the B.M.R. as calculated from the urinary pigment-creatinine ratio is at least as accurate as that calculated from the respiratory calorimeter in 95.4 per cent of instances when cases in which the pigment-creatinine ratio would not be expected to be accurate are eliminated indicates that the pigment-creatinine B.M.R. should be used to check the oxygen consumption B.M.R. when the latter does not seem to agree with the clinical picture. In addition, there is a group (58 cases or 9.8 per cent of patients having metabolism tests) in which the B.M.R. calculated by the pigment-creatinine ratio should be used in preference to the B.M.R. determined by oxygen consumption. We were able to break down this group into five types:

1 Cases of hyperthyroidism under prolonged therapy in which the B.M.R. remains elevated in spite of clinical improvement—10 cases.

*Example:* A. E., a 48-year-old white man, was seen in the Outpatient department for the first time in December, 1945, complaining of weakness, nervousness, palpitations, and weight loss for one year. Physical examination revealed an enlarged firm thyroid gland, warm moist skin, and a tachycardia. B.M.R. was +50. He was given thiouracil and later changed to propylthiouracil in adequate dosage. The patient showed gain in weight and disappearance of all signs of thyrotoxicosis except that repeated B.M.R. determinations remained between +30 and +48. The patient volunteered this information, "When taking

a B.M.R. I'm always nervous and feel as if I'm choking.' In July, 1948, it was decided to stop all antithyroid medication as the patient was asymptomatic although the B.M.R. was +30. In November, 1948, while the patient was still clinically euthyroid, the oxygen consumption B.M.R. was +32 while the B.M.R. calculated on the basis of urinary pigment-creatinine ratio was successively -4 and +2. In January, 1949, while still asymptomatic, the oxygen consumption B.M.R. was +36 while the B.M.R. calculated from the P/C was -8.

## 2. Cases in which an anxiety state or neurocirculatory asthenia causes an abnormally elevated B.M.R. inconsistent with the clinical findings—32 cases.

*Example A:* S. C., a 47-year-old white woman, was admitted to the hospital March 3, 1949. She gave a three-year history of nervousness, hypertension, palpitations, heat intolerance, and sweating. In December, 1946, her B.M.R. was +50 and a subtotal thyroidectomy was performed. Her symptoms persisted for eighteen months postoperatively. In July, 1948, she had a B.M.R. of +7 with all the symptoms of thyrotoxicosis still present. Her present admission was due to the appearance of a small nodule on the left side of the thyroid gland. She showed no evidence of weight loss; there was no tremor; the pulse rate was 100. She was quite apprehensive about the prospect of further surgery. On March 9, 1949, the oxygen consumption B.M.R. was +24 whereas the P/C B.M.R. was -11. On March 15, the oxygen consumption B.M.R. was +21 while the P/C B.M.R. was -13. On March 16, the patient was reassured that no surgery would be necessary. She slept well that night and on the following day the oxygen consumption B.M.R. was +7 and the P/C B.M.R. was -8. The patient was discharged from the hospital with the diagnosis of menopause, essential hypertension, and anxiety state. This patient had received no specific therapy at any time during her hospital stay.

*Example B:* G. A., a 27-year-old white woman, was admitted to the hospital in January, 1949, for an evaluation of an asymptomatic hypertension known to be present for two months. The only significant finding on physical examination was a blood pressure of 200/140. On January 19, a B.M.R. was reported as +34; the P/C B.M.R. calculation was -7. On January 25, a repeat oxygen consumption B.M.R. was -1; the P/C B.M.R. at this time was -2. There was no specific therapy given during the hospital stay. The patient was discharged with a diagnosis of essential hypertension.

*Example C:* E. W., a 30-year-old white woman, was referred to the private office of one of the authors (J. J. V.) for the first time in April, 1949, because she had been found to have a B.M.R. of +60 two weeks previously. She complained of palpitations and a 22 lb. weight loss in the past twenty months following a pregnancy. Physical examination revealed a warm moist skin, thyroid moderately enlarged, pulse rate 128 per minute, and slight prominence of the eyes. A B.M.R. at this time was +47. The patient was started on cyclohexylmethylthiouracil, 300 mg. per day. With the institution of therapy, the patient began to feel better and gain weight, and the pulse rate fell although the B.M.R. remained elevated. Five weeks after she was first seen, she had no complaints, had gained 14½ lb., and the pulse rate was 72 per minute. A B.M.R. at this time was +33. Because of the discrepancy between the clinical condition of the patient and the oxygen consumption B.M.R., a P/C B.M.R. was determined and found to be -3.

## 3. Cases in which it is not technically possible to obtain an accurate B.M.R. (e.g. edentulous patients, patients with punctured eardrums, air swallowers)—5 cases.

*Example:* G. S., a 67-year-old white woman, complained of nervousness. There was no evidence of hyperthyroidism clinically, but because the patient was edentulous, numerous attempts to do a basal metabolism test were unsuccessful. In October, 1948, two successive P/C B.M.R. determinations were +3 and -2.

## 4. Cases in which a basal metabolism test is done on a patient for the first time as part of a routine work-up and yields a high result where there is no evidence of hyperthyroidism—10 cases.

*Example:* S. F., a 56-year-old white woman, came to the Outpatient department complaining of pains in the knees, which were diagnosed as due to osteoarthritis. She was 58 inches tall and weighed 202 pounds. Aside from the obesity and arthritis, the physical examination was negative. A routine oxygen consumption B.M.R. was +30. The P/C B.M.R. was +8.

5. Cases in which the patient refuses to undergo a metabolism determination—1 case.

*Example:* I. C., a 22-year-old white woman, was known to be a diabetic for four years. She had had numerous hypoglycemic reactions. She was hospitalized because of frequent episodes of nervousness and sweating which her physician attributed to an anxiety state. She became very upset at the prospect of undergoing a basal metabolism test and refused it. A B.M.R. calculated on the urinary pigment-creatinine ratio was -3. This result confirmed her physician's impression.

#### SUMMARY

The results of the comparison of the basal metabolic rate as determined by the oxygen consumption method and by the urinary pigment-creatinine ratio method (P/C) in 740 observations made on 424 patients are reported. Of the 740 observations, 457 (61.7 per cent) were on euthyroid patients, 168 (22.8 per cent) were on hyperthyroid patients, and 115 (15.5 per cent) were on hypothyroid patients. The clinical material consisted of hospital, private, and out-patient department patients.

The B.M.R. as determined by the pigment-creatinine ratio (P/C) is unreliable in cases of hypothyroidism, hyperbilirubinemia, azotemia, and in cases in which the urine is discolored by drugs or foods. There were 147 such cases in this series. If these observations are eliminated, then the P/C B.M.R. is at least as accurate as the oxygen consumption B.M.R. in 95.4 per cent of the remaining cases.

The P/C B.M.R. should be used to check all cases in which the oxygen consumption B.M.R. does not agree with the clinical findings in euthyroid and hyperthyroid patients.

The P/C B.M.R. determination should be used in preference to the oxygen consumption B.M.R. for the following groups of patients (98 per cent of our series).

1. Cases of hyperthyroidism under prolonged therapy in which the B.M.R. remains elevated in spite of clinical improvement.
2. Cases in which anxiety state or neurocirculatory asthenia causes an abnormally elevated B.M.R. inconsistent with the clinical diagnosis.
3. Cases in which it is technically impossible to obtain an accurate B.M.R.
4. Cases in which a basal metabolism test is done on a patient for the first time and yields a high result where there is no evidence of hyperthyroidism.
5. Cases in which the patient refuses to undergo a metabolism determination.

The authors appreciate the technical assistance rendered by Mrs. Jennie Shatton, Miss Edna Arzt, and Miss Virginia Reclnitzer, and are grateful to Mr. Jules Joskow, Department of Economics, The City College of New York, for his statistical analysis of the results.

## EFFECT OF RETAINED BRONCHIAL LIPIODOL ON BLOOD IODINE

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NUMEROUS studies have been made on the metabolism of iodine and blood iodine levels in normal people and in various pathologic conditions. Normal standards for protein-bound blood iodine have been established,<sup>1, 2, 3</sup> 3.0 to 8.5  $\mu\text{g}$  per 100 ml. of blood being the quoted normal range. In our laboratory, 5 to 7  $\mu\text{g}$  per 100 ml. is considered normal. Patients with pulmonary tuberculosis have normal protein-bound blood iodine values.<sup>4</sup> Normal blood iodine values have been secured in numerous other pathologic conditions. Most observers agree that hyperthyroidism produces an elevation of the protein-bound blood iodine.<sup>1, 2, 5-8</sup> Turner<sup>2</sup> found elevated blood iodine levels, up to 18  $\mu\text{g}$  per 100 ml. of blood, in lymphatic leucemia. Several observers<sup>1, 6</sup> have found increased protein-bound blood iodine values in patients with liver and gall bladder disease. The effects of previously administered iodine-containing compounds such as gall bladder dyes must be always borne in mind in these latter patients. It is known that the liver takes up iodine and excretes it into the bile. Cohn and Feldman<sup>5</sup> quote Greene and Bruger as finding normal blood iodine values in liver and gall bladder disease, if no iodine-containing compounds had been taken previously. Cohn and Feldman<sup>5</sup> found experimentally that common bile duct ligation in cats failed to produce an elevation of blood iodine. The administration of gall bladder dyes, however, elevates the protein-bound blood iodine for several weeks. A group of patients with advanced liver disease was compared with a group of normal subjects, and blood iodine levels were found to be similar. Rabbits had no elevation of blood iodine following carbon tetrachloride poisoning to produce liver cell necrosis and extensive liver damage.<sup>5</sup>

It is quite clear that an elevated blood iodine may be due to hyperthyroidism and previous administration of iodine-containing compounds, among other things.<sup>7, 8</sup> Chemical laboratories doing blood iodine determinations usually recommend that patients do not receive any iodine-containing compounds for from ten to thirty days prior to the determination of this test.

No information can be found in the literature concerning the quantitative effect on the protein-bound blood iodine of retained Lipiodol in the bronchi and alveoli of the lung as a result of previous bronchograms. Means<sup>9</sup> has noted "the prolonged 'spurious' elevation of protein-bound iodine which follows the administration of such organic iodine-containing compounds as

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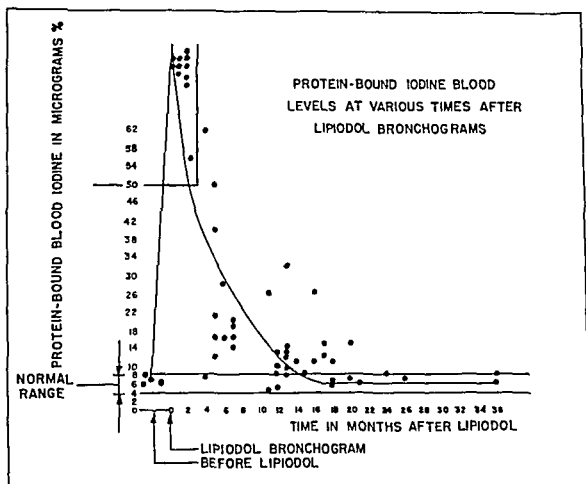


Fig. 1.

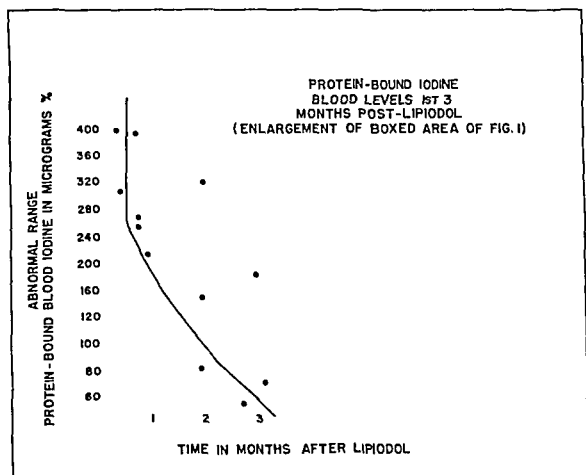


Fig. 2.

Iodeikon or Lipiodol used in roentgenography." Salter<sup>10</sup> has stated that "the intrathecal injection of Lipiodol may form a localized reservoir of iodine which will slowly feed small amounts of iodine into the general circulation over the course of many weeks." Communication with several chemical laboratories and with the manufacturers of Lipiodol indicates that the quantitative effect

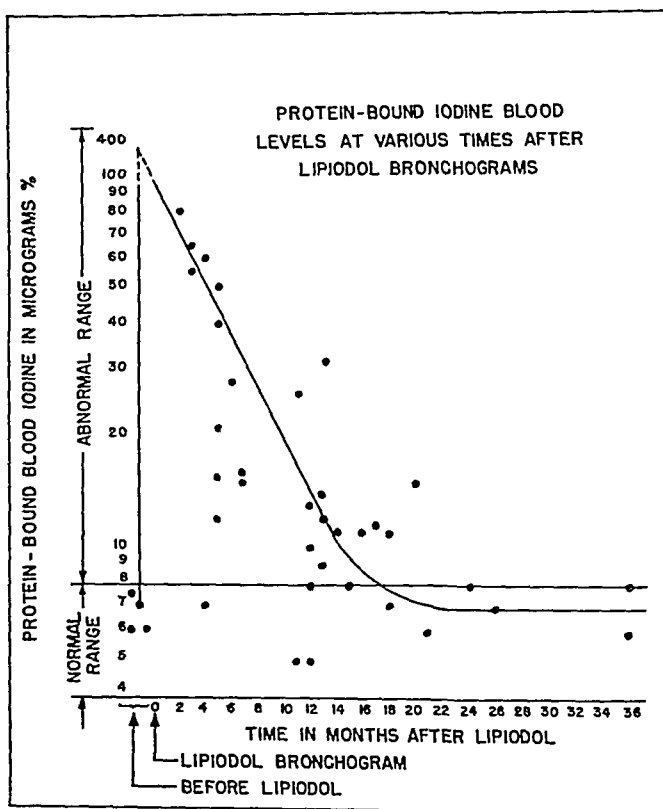


Fig. 3.

of retained Lipiodol is unknown and no studies have been published. The purpose of the present work is to determine the effect of retained Lipiodol upon the blood iodine.

Forty-one determinations of protein-bound blood iodine were secured on thirty patients at varying known intervals following the instillation of Lipiodol\* for a bronchogram. Four patients had normal protein-bound blood iodine determinations before Lipiodol instillation (6, 6, 7, and 8  $\mu\text{g}$  per cent). It soon became apparent that retained Lipiodol causes an elevation of the blood iodine† for prolonged periods of time. Values of over 400  $\mu\text{g}$  per 100 ml. of blood were secured one week following the instillation of Lipiodol. The

\*Lipiodol 40 per cent containing 540 mg. of iodine by weight per milliliter was used. Varying amounts from 5 to 20 ml. were instilled for visualization of the bronchi.

†"Blood iodine" refers to protein-bound blood iodine as determined by the method of Barker,<sup>11</sup> modified by M. E. Morton using 10 per cent trichloroacetic acid to precipitate plasma protein.

protein-bound blood iodine level dropped rather rapidly to average approximately 200  $\mu\text{g}$  per 100 ml. of blood one month after instillation of Lipiodol (still greatly elevated) and then gradually dropped further for some time. It is important to note that the curve drawn of the various values of blood iodine plotted against the time since the last bronchogram did not reach the normal range (4 to 8  $\mu\text{g}$  per 100 ml. blood) until seventeen months after the patient's last bronchogram. (Figs. 1, 2, and 3.)

#### SUMMARY AND CONCLUSIONS

1. Instillation of Lipiodol for a bronchogram produces extremely high levels of protein-bound blood iodine for prolonged periods (months).

2. It requires approximately seventeen months for the blood iodine level to reach normal in patients who have received bronchograms with Lipiodol. Individual patients may have elevated blood iodine values for three and four years.

3. Blood iodine cannot be used as a diagnostic agent (e.g. in suspected cases of hyperthyroidism) in patients who have had Lipiodol bronchograms within the previous two years, and possibly longer.

Grateful acknowledgment is made to Ellen M. Bahr for the blood iodine determinations.

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# THE EFFECT OF THYROID SECRETORY ACTIVITY ON THE DISTRIBUTION OF RADIOIODINE IN PLASMA

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WHEN labelled inorganic iodide is administered by mouth, 90 per cent or more is absorbed in ninety minutes.<sup>1</sup> During this interval, and for several hours thereafter, a portion of the administered dose diffuses into the extracellular fluid. While absorption and diffusion are occurring and during the ensuing twenty-four to forty-eight hours almost all of the iodide ion is competitively removed from the blood and body fluid by the thyroid gland and the kidneys. That which is trapped by the thyroid is soon synthesized into hormone. A variable portion of the latter is stored in the gland while the remainder is secreted into the blood. Once thyroid hormone is released into the blood it apparently undergoes a slow degradation at the rate of about 5 per cent of the existing level per day.<sup>2</sup> Both the rate at which the thyroid gland removes iodine from the blood and the total amount ultimately retained by the gland are characteristically influenced by the state of thyroid activity provided the gland has not been previously saturated with iodine. Each has been measured repeatedly in human subjects by direct and indirect techniques.<sup>3, 4</sup> However, a more positive measurement of thyroid function would necessitate an estimation of the actual rate of hormone secretion. This present study is, therefore, chiefly concerned with the problem of the rate of release of hormone and is based upon serial determinations of radioiodine in the organic fraction of plasma at successive intervals after the administration of a tracer dose of  $I^{131}$ .

## METHODS

Subjects selected for study included patients with either hyperthyroidism or myxedema, along with a group of euthyroid subjects to serve as controls. In none of the patients was the diagnosis equivocal because of grossly atypical clinical findings or inconsistent laboratory data. No subject had received iodine therapy within six weeks or antithyroid drugs within two weeks before the experiments were started.

The tracer dose of  $I^{131}$  was given orally and varied from 0.1 to 0.2 mc. except for occasional patients with hyperthyroidism who received 0.4 millicurie. No normal subjects received more than 0.1 millicurie. The material was either carrier-free or contained not more than 0.5 mg. of NaI. Blood samples were drawn at intervals of two, eight, and twenty-four hours after ingestion of the tracer dose. These were oxalated and a 5 c.c. portion of plasma precipitated with 45 c.c. of Somogyi's zinc sulfate-sodium hydroxide reagent (45 c.c.) as described by Man and associates.<sup>5</sup> After centrifugation, a 20 c.c. aliquot of supernatant was removed and evaporated to dryness in a 2 oz. ointment tin (29 sq. cm. area) by the aid of an infrared lamp. The activity of this fraction was considered to represent that of inorganic iodine.

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TABLE I. PLASMA LEVELS OF I<sup>131</sup> AS PER CENT OF TWO-HOUR INORGANIC LEVEL

INDIVIDUAL VALUES																	MEAN VALUES
HYPERTHYROIDISM																	
PATIENT	L. E.	L. B.*	J. P.	E. N.*	E. J.	E. M.	W. P.	A. P.	P. B.	E. F.	L. P.	E. F.†	I. R.*				
Inorganic fraction	100	3.4	-	48.0	24.0	4.9	3.6	11.0	17.0	11.0	7.2	42.0	4.3	100			
	8 hr.	14.0	0	3.7	20.0	0.5	2.0	3.9	0	1.3	8.4	3.3	0.6	15.7			
	24 hr.	0	-	-	-9	-13	-16	-15	-14	-15	-16	-10	-16	3.9			
	Slope 2-8 hr.	-14	-16	-	-	-	-	-	-	-	-	-	-	-14	±0.69†		
Organic fraction	15.0	25.0	4.0	1.5	2.5	0.8	12.0	33.0	8.0	-	8.7	4.8	4.0	9.9			
	8 hr.	4.1	1.2	-	0.3	2.9	1.9	5.0	5.2	5.6	14.0	1.1	7.7	8.2			
	24 hr.	6.2	4.0	18.0	0.0	2.7	5.4	21.0	8.2	15.0	30.0	2.4	9.2	14.8			
	Slope 8-24 hr.	+0.13	+0.17	-	-0.02	-0.01	+0.22	+1.0	+1.5	+0.19	+0.59	+1.00	+0.08	+0.09	+0.41	±0.14	
NORMAL SUBJECTS																	
PATIENT	A. M.	I. P.	W. F.	M. M.	W. G.	I. L.	G. M.	E. L.	J. B.	A. P.	C. B.						
Inorganic fraction	100	56	47	64	75	69	71	69	31	43	37			100			
	8 hr.	66	15	12	28	17	34	12	28	4	8	3		57.2			
	24 hr.	19	-7	-9	-6	-4	-5	-5	-11	-6	-10			16.3			
	Slope 2-8 hr.	-6	-7	-9	-6	-4	-5	-5	-11	-6	-10			-7	±0.69		
Organic fraction	5.1	2.4	1.6	1.2	8.6	23.0	20.0	9.5	3.3	0.6	15.0			8.2			
	8 hr.	3.0	1.4	1.3	1.8	3.4	11.0	-	11.0	1.3	0.3	9.7		4.4			
	24 hr.	1.7	0.2	0.4	0.8	5.1	3.7	1.2	5.2	0.7	0.1	5.1		2.2			
	Slope 8-24 hr.	-0.08	-0.08	-0.06	-0.06	+0.11	-0.46	-	-0.36	-0.04	-0.01	-0.29		-0.13	±0.05		
MYXEDEMA																	
PATIENT	A. H.	L. J.	J. B.	I. R.	L. H.												
Inorganic fraction	100	57	96	60	105									100			
	8 hr.	68	37	76	22	96								77			
	24 hr.	27	20	76	22	96								48			
	Slope 2-8 hr.	-5.3	-7.2	-0.7	-6.7	0.0								-4.0	±1.5		
Organic fraction	6.2	5.8	6.6	3.8	1.0									4.7			
	8 hr.	4.8	3.0	7.2	2.3	1.6								3.8			
	24 hr.	3.5	1.6	8.8	0.9	1.2								3.2			
	Slope 8-24 hr.	-0.08	-0.09	+0.10	-0.09	-0.02								-0.036	±0.036		

\*Nodular glands (all others are diffuse).

†Standard error of mean.

The protein precipitate which contained organic hormonal iodine was washed three times with 50 c.c. portions of water. Assays of the washings on six different blood samples indicated that any additional washing would not remove more activity than 0.5 per cent of that in the total supernatant fraction from which the precipitate was removed. The washed precipitate was quantitatively transferred to a weighed ointment tin and dried under the heat lamp: It was then distributed evenly over the bottom of the tin with the help of a stirring rod and a small quantity of acetone. The half-density of the precipitate in the containers averaged only 10 mg. per square centimeter.

Counts were made under a Geiger-Müller tube with a 6 mg. per square centimeter mica window, 6.2 cm. in diameter and connected with a scale of 8 counter. An appropriately sized aliquot of the original material was counted at the same time so that decay calculations were unnecessary. Aliquots of the standard and inorganic fraction were combined with 1 c.c. of a solution recommended by the Oak Ridge National Laboratory to prevent loss of volatile iodine.\*

### RESULTS

Individual values are recorded in Table I, and composite curves of the inorganic and organic fractions are depicted in Fig. 1. The value of the inorganic fraction at two hours was arbitrarily set at 100 per cent as reference for the remaining determinations. Since total blood or body iodine content was not known, there was no advantage in employing the original dose as a standard for comparison.

The inorganic fraction is seen to decline much more rapidly in hyperthyroid patients than in normals. This is presumed to be due to the more rapid rate of removal by the thyroid gland, provided there is no significant increase in the rate of renal removal. It is of interest that the decrease of plasma iodine with time in the three curves does not give a straight line of constant slope on a semilogarithmic plot. Without more points on the curve it is impossible to come to any exact conclusions, but it may well be that this reflects the sequential operation of several biologic processes, each in itself expressed by a true exponential function.

A more rapid decline is present between the second and eighth hour than between the eighth and twenty-fourth. The initial rapid fall could be due to two factors. (1.) The passage of iodine into the extracellular fluid in addition to the thyroid and the urine. (2.) An exaggerated peak of the blood concentration if the rate of absorption should materially exceed the rate of diffusion into the extracellular fluid. The slope of the three curves between the second and eighth hour when inorganic iodine is being disposed of most rapidly may be seen in the table and in Fig. 1. The difference between hyperthyroid and normal subjects is very real. However, the five patients with myxedema, although showing a flatter average curve, do not differ from the normals to a statistically significant degree.

The behavior of the organic fraction likewise is not the same in hyperthyroid subjects as in normals. Between the eighth and twenty-fourth hour the patients with hyperthyroidism show a distinct rising trend, while the curve of the normal subjects tends to decline. This difference in behavior of the two curves is a real one, as an analysis of the slopes of the two groups

\*NaHSO<sub>3</sub>, 500 mg.; NaI, 250 mg.; NaOH, 750 mg.; water to 1,000 c.c.

will reveal (Table I). The trend of the organic fraction in myxedematous patients is not significantly different from that of the normal group. An observation to be discussed later is the high activity which was encountered in the organic fraction at the two-hour period.

#### MEAN PLASMA RADIOACTIVE IODINE LEVELS

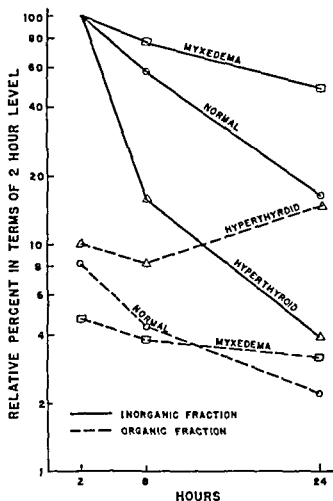


Fig. 1.

In four of the hyperthyroid patients and four normals additional measurements were made after forty-eight hours. The trend of the organic and inorganic curves during this interval was similar to that observed between the eighth and twenty-fourth hour.

#### DISCUSSION

McConahey, Keating, and Power have recently reported their results of blood fractionation studies in a small series of patients and normal subjects who were given doses of  $I^{131}$  ranging from 1 to 100 millicuries.<sup>6</sup> Our own findings are similar in some respects to those reported by this group of workers. The chief point of difference is that the organic fraction in their three normal patients and also in the one hypothyroid patient showed a progressive rise, albeit with a more gradual slope than in the series of hyperthyroid cases. The curves for both normal and hypothyroid patients presented here show a slight progressive fall in activity of this fraction. The reason for

this decline cannot be determined with any degree of precision; however, certain general principles should be noted. There is a slow process of disintegration of thyroid hormone in the body fluids which should proceed at approximately equivalent rates in all subjects. In hyperthyroidism, with relatively little hormone stored in the gland and a relative high uptake of radioiodine, it is to be expected that the material secreted after a tracer dose would be proportionately rich in radioactive iodine. On the other hand, with a normal gland, the comparatively large amount of preformed, nonradioactive hormone would force the gland to deliver to the blood a product relatively poor in the newly acquired radioactive component. Thus it is conceivable that in its normal state of activity the quota of radioactive hormone delivered to the blood would be insufficient to result in a steadily mounting activity within the organic fraction. The rate of change of organic iodine in the blood, therefore, does not offer a direct measure of true secretory activity. That the behavior of the organic fraction is quantitatively influenced by thyroid hyperactivity is nevertheless clearly demonstrated by the difference in slope of the curves as shown here.

The finding of small but unmistakably real quantities of active material in the organic fraction of patients with myxedema in the present study, and in that of McCahey and co-workers, is of interest. Moreover, our own finding of a relatively high value as soon as two hours after ingestion with a decline during the next six hours even in the presence of hyperthyroidism should also be commented upon. An explanation of the presence of active material in myxedematous patients, along with the temporarily high level in all patients, might suggest an extrathyroidal synthesis of an organic iodine complex. Incubation of serum with inorganic iodine under physiologic conditions has not been found to promote such a synthesis.<sup>6</sup> Morton and co-workers, on the other hand, have demonstrated the *in vivo* production of thyroxine and diiodotyrosine in thyroidectomized rats as early as two hours after the administration of labelled inorganic iodide.<sup>7</sup> It is difficult to believe, however, that the high level of serum activity here encountered at two hours could be achieved so rapidly by extrathyroidal synthesis. Moreover, the initial drop in the curve (between two and eight hours) is too rapid to be attributed to simple thyroxine decay. There is a possibility that a portion of the protein-bound material is not actually a hormonal compound but that it is fixed to protein fully as firmly and resists removal by washing.

The usefulness of a determination of blood radioiodine as a diagnostic test cannot be determined on the basis of the present observations. Significant differences between mean values can easily be demonstrated, but an overlap exists. The steepest slope for the curves of inorganic iodine (two to eight hours) in the normal group was -11 (Table I). However, two hyperthyroid patients (E. N. and E. F.) showed slopes which were less steep. Both patients suffered from a relatively chronic type of disease of only moderate severity and each had a rather large nodular gland. In the case of the organic fraction, if one arbitrarily divides hyperthyroid patients from normals on the basis

of a positive or negative slope between eight and twenty-four hours, the overlap consists of one normal patient with a positive slope and two hyperthyroid patients with slightly negative slopes.

Thus the large majority of our cases show definite differences between normal and hyperthyroid blood values. Just how much overlap actually exists can be shown only by a large series. It may well be that this type of determination will prove a useful adjunct to already existing tests for hyperthyroidism.

Freedberg and co-workers have recently reported that protein-bound radioiodine, twenty-four hours after a tracer dose, was consistently higher in hyperthyroid patients than in normal controls.<sup>8</sup> In the present study the rate of change between eight and twenty-four hours correlated with clinical status more consistently than did the final twenty-four hour level.

The behavior of the inorganic fraction in myxedema was not greatly different from normal and the rate of change of the organic component was not distinguishable from that in the normal group. In our own experience the best diagnostic criterion for myxedema when one employs radioiodine techniques is the failure to demonstrate any local uptake of activity by direct measurement over the gland with a counter.

#### SUMMARY

After a tracer dose of radioiodine the activity of the inorganic fraction of the blood in hyperthyroidism declines more rapidly than normal, while in myxedema the decline tends to be somewhat slower than normal. The activity of the organic fraction is distinctly elevated in all types of cases at the end of two hours, and then tends to decline between the second and eighth hour. This decline is progressive for the ensuing sixteen hours in both normal subjects and patients with myxedema; however, in patients suffering from hyperthyroidism there is characteristically a progressive rise in this fraction between the eighth and twenty-fourth hour.

We are indebted to Ethel Buchwald Chudziek for technical assistance.

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# INHIBITION OF THE GROWTH OF STAPHYLOCOCCUS AUREUS BY HUMAN SEMEN

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THE presence of penicillin in blister fluid of human skin following parenteral and topical application was determined by Dostrovsky, Gurevitch, and Rozansky.<sup>1</sup> In another study the concentration of penicillin in human milk was determined by Rozansky and Brzezinsky.<sup>2</sup> In pursuing these studies further we attempted to determine whether penicillin is excreted in the human semen. It was first necessary to find out whether semen in itself has any inhibitory effect on the growth of bacteria. Preliminary examinations revealed inhibition of the growth of *Staphylococcus aureus* by human semen. The following is a detailed report on a study of the inhibition of the growth of *Staph. aureus* by human semen.

## MATERIALS AND METHODS

Twenty-eight specimens of semen obtained from twenty-four men between the ages of 27 and 47 years were examined. The semen of four patients was examined twice. Five of the examined specimens were azoospermic, ten were oligospermic (spermatozoa count lower than 60,000,000 per cubic centimeter), and thirteen were normospermic. The patients applied to the clinic for consultation in connection with sterile marriage. Only one patient (Specimen 21) had received 60 mg. testosterone propionate (six injections of 10 mg.) before examination. All the others had received no previous treatment. In most instances the semen was obtained under as sterile conditions as possible by masturbation in the outpatient clinic, but in four instances specimens were brought to the clinic from outside. Each specimen was tested for bacterial contamination before examination. One specimen was discarded because of contamination by *Esch. coli*. In five other ejaculates a few colonies of *Staphylococcus albus* were found.

The inhibitory effect of human semen on bacterial growth was examined by the cup method used in studies on the concentration of antibiotics.<sup>3</sup> The first test strain was *Staph. aureus*, Heatley, in use in antibiotic studies in this laboratory for a period of four years. In the course of later experiments two additional strains of *Staph. aureus* freshly isolated in the laboratory were used: strain 924 isolated from the urine of a patient with cystopyelitis, and strain 598 isolated from the pus in a case of furunculosis. All three strains had the usual properties of pathogenic strains of *Staph. aureus*: coagulase positive, mannite positive, actively hemolytic.

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Examinations were conducted as follows. One cubic centimeter of a 1:1,000 dilution of a twenty-four hour broth culture of the test strain was added to 20 c.c. of nutrient agar (Difco) and plates were poured. The plates were left at room temperature for four hours, after which five cups, 8 mm. in diameter (external), were placed on each plate. Four cups each received 3 drops of the tested ejaculates and the fifth received 3 drops of a penicillin solution containing 0.1 unit per cubic centimeter. The cup containing penicillin served as a control of the area of inhibition of growth of the staphylococci. The results were read after twenty-four hours of incubation at 37° C. In most examinations two plates were prepared for each specimen examined. The specimens were left at room temperature for four hours before examination to permit the ejaculates to liquify and to become homogenous. When sufficient material was available, residues were stored at 8° C. after the first examination and were re-examined twenty-four hours to seventy-two hours later.

### RESULTS

The results of the examinations are summarized in Tables I, II, and III, comprising studies in the Heatley strain, strain 924, and strain 598 respectively.

As may be seen in Table I, twenty-three of the twenty-eight specimens studied with the Heatley strain inhibited the growth of the staphylococcus over an area from 10 to 20 mm. in diameter, while five gave negative results. The positive results include examinations repeated after twenty-four and seventy-two hours. In three instances no inhibition was found at the first examination but the same specimens were positive when examined twenty-four and seventy-two hours later. Of the twenty-three positive specimens, fourteen yielded similar areas of inhibition in both plates and nine specimens yielded positive results in one plate (four of these specimens were examined with one plate only). Specimen 17 was examined in three plates, and areas of inhibition 16 mm. in diameter were observed in all of them. In six instances the semen was re-examined following twenty-four hours of storage at 8° C. Five of these specimens were positive and one was negative. Three of the positive specimens were particularly interesting because they were negative on first examination but yielded a distinct area of inhibition twenty-four and seventy-two hours later. Following two weeks of storage at 8° C. Specimen 14 yielded an area of inhibition similar to that of its first examination. Specimen 21 was negative following one week's storage at 8° C.

The inhibitory effects of eighteen specimens of human sperm on *Staph. aureus* strain 924, isolated from urine, are summarized in Table II.

Fourteen of the eighteen specimens were positive, five in two plates and nine in one plate. Four specimens in this series were negative.

The inhibition of *Staph. aureus* strain 598, isolated from a furuncle, by eight specimens of human sperm is summarized in Table III. Of the eight examinations five were positive, each of them in two plates, and three were negative when examined in one plate only.



TABLE I. INHIBITION OF GROWTH OF STAPH. AUREUS, HEATLEY STRAIN, BY HUMAN SEMEN

SPECIMEN	AGE OF PATIENT	QUANTITY OF SEMEN (C.C.)	SPERMATOZOA (MILLIONS/C.C.)	ZONE OF INHIBITION IN MM. AFTER				
				4 HR.		24 HR.	72 HR.	
				PLATE I	PLATE II	PLATE I	PLATE I	PLATE II
1	27	1	Azoospermia	17	-	-	-	-
2	25	2	80	14	14	-	-	-
3	30	3	2	19	19	-	-	-
4	29	4	70	11	0	-	-	-
5	32	3	120	11	0	-	-	-
6	38	1	85	14	-	-	-	-
7	29	3	40	10.5	0	-	-	-
8	37	4	30	14	0	13	-	-
9	44	3	6	0	0	12	-	-
10	47	2.5	130	0	0	-	-	-
11	39	8	1	0	-	0	0	0
12	33	3	Azoospermia	0	0	15	12	11
13	27	6	70	0	0	15	15	15
14	39	4	40	14	10	-	-	-
15	26	3	20	0	0	-	-	-
16	28	3	70	0	-	-	-	-
17	30	2	Azoospermia	16	16	-	-	-
18	38	3	60	10	10	-	-	-
19								
(1, on second examination)	27	1	Azoospermia	0	0	-	-	-
20								
(17, on second examination)	30	1.5	Azoospermia	16	15	-	-	-
21	44	2	50	16	15	-	-	-
22	32	3	45	15	12	-	-	-
23	35	3	70	11	0	-	-	-
24	42	4	35	16	15	-	-	-
25	41	3	108	17	16	-	-	-
26	35	3	80	13	-	-	-	-
27								
(6, on second examination)	38	2	85	11	9	15	-	-
28								
(18, on second examination)	38	5	65	20	15	-	-	-

-, Not examined.

0, No inhibition.

TABLE II. INHIBITION OF GROWTH OF STAPH. AUREUS, STRAIN 924, BY HUMAN SEMEN

SPECIMEN	ZONE OF INHIBITION IN MM. AFTER 4 HR.	
	PLATE I	PLATE II
10	11	-
11	18	-
12	10	-
13	11	-
14	15	10
15	0	-
16	0	-
17	14	14
18	0	-
19	0	0
20	20	15
21	12	12
22	11	0
23	15	-
24	12	0
25	14	13
26	10	-
28	15	0

-, Not examined.

0, No inhibition.

TABLE III. INHIBITION OF GROWTH OF STAPH. AUREUS, STRAIN 598, BY HUMAN SEMEN

SPECIMEN	ZONE OF INHIBITION IN MM. AFTER 4 HR.	
	PLATE I	PLATE II
14	12	11
16	17	17
17	12	12
20	14	11
21	12	12
22	0	-
23	0	-
28	0	-

-, Not examined.

0, No inhibition.

## COMMENT

Human semen has been found capable of inhibiting growth of several strains of *Staph. aureus*. Inhibition was observed in 80 per cent of the examinations done with three strains of *Staph. aureus*. The active principle of the semen was not associated with the spermatozoa, since only the supernatant fluid of the semen was used in the experiments. This is supported by the fact that four of the azoospermic specimens showed inhibitory activity. At this stage of the investigation little can be said as to the nature of the active principle. The factor is stable when stored for days at 8° C. Specimen 14 remained active even after fourteen days. The active principle is thermostable. Specimen 18 remained positive after heating for half an hour at 56° C. Specimens 24 and 25 retained their activity after exposure to 75° C. for fifteen minutes.

Three specimens were negative in the first examination but found to be positive in examinations repeated after twenty-four and seventy-two hours. It may be of interest that these three specimens were not kept at room temperature as is usual in these experiments, but were placed mistakenly in the refrigerator when brought from the outpatient clinic. It may be assumed that the homogenization of these specimens, which takes place at room temperature, was interfered with by refrigeration.

Six specimens of sperm which inhibited *Staph. aureus* had no effect on the growth of *Esch. coli*.

## SUMMARY

Twenty-eight specimens of human semen were tested for their inhibitory effects on the growth of *Staph. aureus*. Eighty per cent of the specimens inhibited the growth of this organism. Preliminary studies indicate that the active principle is associated with the liquid part of the semen. Some properties of the active principle in the semen are discussed.

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# OBSERVATIONS ON THE USE OF A NEW ANALGESIC, NU-2206 (3-HYDROXY-N-METHYLMORPHINAN HYDROBROMIDE)

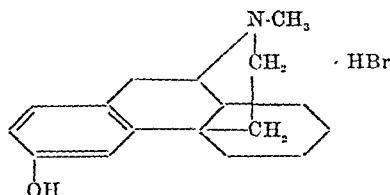
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## INTRODUCTION

THIS is a preliminary report on a clinical study of the analgesic properties of Nu-2206\* (3-hydroxy-N-methylmorphinan-hydrobromide) which includes observations on a series of normal subjects, on a series of patients with postoperative pain, and on a series of patients with intractable pain due to cancer. In instances abstracted in this report it was possible to observe the effect of this drug during and after prolonged use.

## CHEMISTRY

Nu-2206 (3-hydroxy-N-methylmorphinan-hydrobromide) has the following structural formula:<sup>1</sup>



Nu-2206 occurs in the form of colorless crystals melting at 193° to 195° C. The substance is rather soluble in water at 20° C. It is easily soluble in water at 100° C. and in alcohol, but it is insoluble in ether. It has analgesic properties similar to those of morphine and some of the other morphine-like compounds developed recently.<sup>2, 3</sup>

## ADMINISTRATION TO NORMAL SUBJECTS

The effect on the pain threshold in six normal volunteer student subjects was determined by the Hardy-Wolff-Goodell technique. Doses used were 0.5, 1.0, 2.0, and 3.0 milligrams. Each student received two subcutaneous doses of each of the four graded doses. No subject received more than two doses each week. These doses were compared with doses of 5.0, 10.0, and 15.0 mg. of morphine sulfate given in similar manner and under similar conditions. The results indicated that Nu-2206 was about four times as potent as morphine on a per milligram basis. Peak threshold effects were reached at about the same time as with morphine but there was a somewhat greater duration of effect. The blood pressure changes were no greater than could be accounted for by sedation and resting of the subject.

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Doses of 0.5 to 1.0 mg. apparently produced no symptoms in any of the volunteers other than awareness of some vague gastrointestinal distress. Slight sedation may have occurred in some instances. Doses of 2.0 mg. produced some nausea (but no vomiting), dizziness, and sedation. The subjects were aware of gastrointestinal contraction and some inability to concentrate. Doses of 3.0 mg. produced some nausea in practically all subjects; vomiting occurred in two different subjects on one occasion each and dizziness occurred in all subjects. Sedation, inability to concentrate, and awareness of gastrointestinal symptoms were experienced by all subjects. In one subject vomiting occurred five to six hours after the injection. No euphoria was experienced by any of the subjects. In general, 3.0 mg. of Nu-2206 produced fewer and less severe symptoms than an equivalent analgesic dose of morphine. All subjects on 3.0 doses stated they either went to bed right after dinner (five to six hours after injection) or were too sedated to do any studying during the course of the evening. Respiration was not studied, but by casual observation no significant respiratory changes were noticeable.

#### NU-2206 IN THE TREATMENT OF POSTOPERATIVE PAIN

Nu-2206 was used subcutaneously in a total of fifty-eight postoperative urological and general surgical patients. Thirty-six patients (twenty-nine urological and 7 general surgical) were given 1.5 milligrams. Twelve urological patients were given 4.5 milligrams. Six general surgical patients were given

TABLE I. COMPARISON OF ANALGETIC POTENCIES UPON SUBCUTANEOUS INJECTION

DRUG	DOSE (MG.)	NUMBER OF DOSES	HEIGHT PEAK % RISE THRESHOLD $M \pm \sigma^*$	TIME TO REACH PEAK $M \pm \sigma^*$ (MIN.)	DURATION TO 10% OF PEAK $M \pm \sigma^*$
Morphine SO <sub>4</sub>	10	6	$21.5 \pm 1.9$	$73.3 \pm 8$	$246 \pm 33.2$
Morphine SO <sub>4</sub>	15	5	$27.2 \pm 2.5$	$84.0 \pm 7.7$	$280 \pm 7.4$
Nu-2206	1	11	$10.1 \pm 3.0$	$65.0 \pm 9.3$	$211 \pm 13.9$
Nu-2206	2	9	$18.2 \pm 2.1$	$82.0 \pm 5.9$	$267.0 \pm 21.4$
Nu-2206	3	9	$24.8 \pm 2.9$	$91.1 \pm 8.3$	$301 \pm 27.2$

\* $M \pm \sigma$  is the mean effect  $\pm$  its standard deviation.

3.0 milligrams. Four general surgical patients were given 6 milligrams. The majority of the patients to whom 1.5 mg. were given had transurethral resections. These patients usually have very definite discomfort and in some cases severe pain. Ordinarily these patients are given 10 mg. of morphine sulfate immediately after completion of the procedure as the spinal anesthesia is wearing off, and the same dosage is usually repeated in several hours. In these patients Nu-2206 in a dosage of 1.5 mg. was uniformly ineffective. In the patients who had other miscellaneous urologic procedures—three nephrectomies, two suprapubic cystotomies, and one orchidopexy—there was also failure to relieve pain. The one good result in urological patients was in a 9-year-old boy who had a nephrectomy. In the general surgical patients in this dosage range, four obtained relief and three obtained no relief. These seven patients represented a variety of operations. It may be that these surgical procedures did not result

in as much postoperative pain as the average urologic procedure. It appeared that 1.5 mg. of Nu-2206 was an unsatisfactory dose.

In six instances Nu-2206 was used in dosage of 3.0 mg. for treatment of postoperative pain in general surgical patients. It afforded relief in three instances and gave insufficient relief in the remaining three. One of the latter patients subsequently obtained relief of pain with 6 milligrams.

Eleven urological patients who had had transurethral resection were given fifty-one 4.5 mg. doses. However, in this series, Nu-2206 was not given unless the patient had real pain and requested relief. In this dosage range, which was felt to be roughly equivalent to 15 mg. of morphine, Nu-2206 was uniformly effective in relieving pain. Demonstrable effect was noted in most instances thirty minutes after administration of the drug. Analgesic effect was present longer than that observed with morphine and in most instances it was effective for six hours or more. There were no undesirable side effects.

Six milligram dosage was employed in four general surgical patients. In all of the patients to whom 6.0 mg. were given, excellent relief was obtained. The following detailed case is presented as an example of this series.

Patient M. W. (48-9796), a 25-year-old man, was admitted on Aug. 8, 1948, following multiple injuries received in an auto racing accident. Among his injuries were: a perforating wound of the abdominal wall, peritonitis, traumatic rupture of the spleen, severe contusion of the colon, fractures of the transverse processes of the third and fourth lumbar vertebrae, and fracture of left zygoma. Surgical procedures included a splenectomy and colostomy.

TABLE II. PATIENT M. W.

DATE	TIME	DRUG	REMARKS
8/31/48		100 mg. Demerol 5 times per day	Relief
9/ 1/48		100 mg. Demerol 5 times per day	Relief
9/ 2/48		100 mg. Demerol 5 times per day	Relief
9/ 3/48	0300	3 mg. Nu-2206	No relief
	1045	1 mg. Nu-2206	No relief
	1630	3 mg. Nu-2206	No relief
	2000	3 mg. Nu-2206	No relief, <i>emesis</i> , excessive perspiration
9/ 4/48	0215	3 mg. Nu-2206	Slept for more than 3 hr.
	1700	3 mg. Nu-2206	Relief from pain for about 3 hr.
	2230	3 mg. Nu-2206	Good relief
9/ 5/48	0230	3 mg. Nu-2206	Slept for 2½ hr.
	0800	3 mg. Nu-2206	Relief
	1520	3 mg. Nu-2206	Relief for about 2 hr.
	2050	3 mg. Nu-2206	Slept more than 2 hr.
9/ 6/48	0030	3 mg. Nu-2206	Slept about 2½ hr.
	0500	3 mg. Nu-2206	Relief
	1545	3 mg. Nu-2206	No relief
	2330	3 mg. Nu-2206	Slept more than 2 hr.
9/ 7/48	0600	3 mg. Nu-2206	Asleep within ½ hr.
9/ 8/48	0345	3 mg. Nu-2206	Asleep within 15 min.

Control of pain was sought by 30 mg. of morphine every four hours during the early stages of hospitalization. This dose was reduced as circumstances allowed, and on August 30, 100 mg. Demerol were substituted for morphine. A tabulation of the drugs given this patient is presented in Table II.

## NU-2206 IN THE TREATMENT OF INTRACTABLE PAIN IN CANCER

Five patients with intractable pain due to cancer were observed for varying periods of time up to six months. The following case is presented in detail and is illustrative of some of the effects observed during a prolonged course of administration.

Patient G. M., a 55-year-old white woman, was admitted to the University Hospitals on Oct. 28, 1947, with a diagnosis of carcinoma of the cecum. At operation a carcinoma of the ascending colon with regional lymph node metastasis was found and a right hemicolectomy was performed. She returned to the hospital on Jan. 3, 1949, with a large mass in the right upper quadrant of the abdomen which appeared to be liver involved by metastatic carcinoma. A posteroanterior radiogram of the chest revealed elevation and nodular outline of the diaphragm. The patient had been having very severe pain for two weeks and her physician had been unable to keep her free of pain by intermittent injections of morphine sulfate and Demerol. At the time of her admission she was emaciated and was apparently losing ground rather rapidly, although she was still able to be out of bed for short periods of time. A right splanchnic block was done; this afforded temporary relief of the pain. This was repeated

TABLE III. PATIENT G. M.

DATE	TIME	DRUG	RESULTS
1/ 8/49	1215	6 mg. Nu-2206	Pain relieved in 45 min.
	2015	6 mg. Nu-2206	Pain relieved in 1½ hr.
1/ 9/49	1615	6 mg. Nu-2206	Pain relieved in 45 min.
1/10/49	1700	6 mg. Nu-2206	Pain partially relieved in 30 min.; completely relieved in 60 min.
			Before: pulse 128, resp. 28
			After: pulse 124, resp. 24
1/11/49	1100	6 mg. Nu-2206	No record of result
	2030	6 mg. Nu-2206	Sleeping soundly in 2½ hr.
			Before: pulse 112, resp. 24
			After: pulse 96, resp. 20
1/12/49	0655	6 mg. Nu-2206	Sleeping soundly at 0730
1/13/49	1415	150 mg. Demerol	1515, pain relieved. Almost asleep
1/16/49	0930	150 mg. Demerol	1000, sleeping at intervals
1/17/49	1000	6 mg. Nu-2206	45 min., stated she was not relieved. Later in day stated she spent a comfortable afternoon
			Before: pulse 120
			After: pulse 112
1/21/49	1930	6 mg. Nu-2206	2010, asleep
	1920	6 mg. Nu-2206	2045, no pain
			Before: pulse 124, resp. 22
			After: pulse 120, resp. 20
	2330	6 mg. Nu-2206	0200, asleep
1/22/49	2000	6 mg. Nu-2206	2100, no pain. 2200, asleep
1/23/49	1930	6 mg. Nu-2206	2000, drowsy. 2100, asleep
1/24/49	2000	6 mg. Nu-2206	2100, asleep
1/27/49	1930	6 mg. Nu-2206	2000, dozing
1/28/49	2000	6 mg. Nu-2206	2100, asleep
1/29/49	1930	6 mg. Nu-2206	2000, asleep. Slept all night
1/30/49	1930	6 mg. Nu-2206	2100, asleep
1/31/49	0545	6 mg. Nu-2206	0600, asleep
	2000	6 mg. Nu-2206	Pulse 130, resp. 36. Pulse thready
	2200	6 mg. Nu-2206	Asleep. Expired 2-1-49, 0145

once without satisfactory analgesia. Cordotomy and prefrontal lobotomy were considered but because of the patient's poor general condition and the short life expectancy, it was deemed advisable to try to obtain relief from pain by using analgesic agents rather than to subject the patient to a hazardous operative procedure. As may be seen from Table III, she received a total of twenty-three doses of 6 mg. of Nu-2206 subcutaneously. This was given from January 8 to January 31 when she expired. The degree of relief accomplished by this means was considerable, and particularly gratifying was the long period of analgesia afforded. It was usually possible for her to sleep throughout the night without awakening with pain. This was in marked contradistinction to what had been observed with 10 mg. doses of morphine and 100 mg. doses of Demerol. In two instances during the period the patient was in the hospital, 150 mg. of Demerol were given and the result appeared to be less enduring than with Nu-2206 in a dose of 6 milligrams.

In one urological patient who had an inoperable retroperitoneal tumor with continuous severe pain, Nu-2206 in repeated doses of 4.5 mg. twice daily for a total of 113.5 mg. in twenty-three doses was given throughout his stay in the hospital. He required no other analgesic drug.

In another patient, a 75-year-old white man (Patient E. T.) who had severe pelvic pain following recurrence and local metastasis of carcinoma of the rectum after abdominal perineal resection, Nu-2206 proved to be very effective in producing analgesia. A total of 111 doses, totaling 353 mg., was given over a period of sixteen weeks. Three milligrams proved sufficient when given at bedtime to produce comfort throughout the night so that the patient did not require other analgesic agents or barbiturates for rest. Occasionally during the daytime he took small doses of aspirin (0.6 Gm.) and in a few instances 60 mg. doses of codeine sulfate. During the first five weeks on Nu-2206, he received only eight doses of codeine. After he had received the drug for seven and one-half weeks, complete withdrawal was done for five days. The patient had no symptoms attributable to withdrawal. Examination five days after withdrawal showed no signs indicating withdrawal effect. Due to severe pain the patient was again placed on the drug and the dose was increased to 5 mg. shortly before death. After fourteen weeks he developed symptoms and then signs indicating subtentorial brain metastasis. Three weeks later he expired. No Nu-2206 was given during the last six days of life because of coma.

A summary of our experience with Nu-2206 in the treatment of pain in patients with cancer is presented in Table IV.

Naturally the question of habituation and withdrawal effects is also raised. In addition to the instance already cited (Patient E. T.) in which withdrawal of the Nu-2206 was done after many weeks, ten other patients were studied in this regard. These all received the drug for much shorter periods. None of them demonstrated withdrawal effects. This experience is summarized in Table V.

TABLE IV. NU-2206 IN TREATMENT OF PAIN OF CANCER

PATIENT	SEX	AGE	INDIVIDUAL DOSAGE	NUMBER OF DAYS ADMINIS- TERED	TOTAL DOSAGE	EFFICACY	SIDE REACTIONS	DIAGNOSIS OR PROCEDURE
C. H.	M	61	6 mg.	4	96 mg.	Maximal	None	Carcinoma of lung (exploratory tho- racotomy)
G. M.	F	55	6 mg.	17	126 mg.	Good-maximal	None	Metastatic carcinoma to liver
I. A.	F	78	1.5 mg.	3	4.5 mg.	Maximal	None	Carcinoma of rectum; metastasis and local recurrence
G. W.	F	26	3.0 mg.	3	15.0 mg.	Slight-moderate	None	Mesothelioma of pleura
R. S.	M	58	4.5 mg.	12	113.5 mg.	Maximal	None	Inoperable retroperitoneal tumor
D.	F	32	4.5 mg.	6	88.5 mg.	Good-maximal	Slight dizziness	Carcinoma of cervix
K.	F	40	2.0 mg.	28	20.0 mg.	Slight-moderate	None	Carcinoma of cervix
T.	F	80	2.0 mg.	4	10.0 mg.	Moderate	None	Carcinoma of vulva
C.	F	61	3.0 mg.	4	30.0 mg.	Maximal	None	Carcinoma of cervix
E. T.	M	75	3.0 mg.	35	108 mg.	Maximal	No undesirable ones	Recurrent and metastatic carcinoma of rectum in pelvis



TABLE V. ABSENCE OF WITHDRAWAL EFFECTS OF NU-2206

PATIENT	SEX	AGE	INDIVIDUAL DOSAGE	NUMBER OF DAYS BEFORE DRUG WITHDRAWN	TOTAL DOSAGE	EFFICACY	SIDE REACTIONS	DIAGNOSIS OR PROCEDURE	WITH- DRAWAL SYMPTOMS
E. H.	F	34	5 mg.	6	130 mg.	Maximal	Nausea and vomiting, two times	Metastatic epidermoid carcinoma of cervix to 2nd, 3rd, 4th ribs, rt.	None
M. M.	F	82	5 mg.	5	60 mg.	Good	Pulse irregular	Tic douloureux.- Nerve section	None
E. S.	M	63	6 mg.	10	84 mg.	Maximal	None	Bronchogenic carcinoma (Rt. pneumonectomy)	None
H. N.	M	53	6 mg.	15	84 mg.	Maximal	None	Metastatic carcinoma in pelvis. Previous abdominal-perineal resection	None
C. B.	M	71	6 mg.	10	78 mg.	Maximal	None	Inoperable carcinoma of lower lobe of left lung	None
E. H.	M	57	5 mg.	2	12 mg.	Maximal	None	Splenectomy for Banti's syndrome	None
E. B.	M	48	5 mg.	7	70 mg.	Maximal	None	Phantom limb pain	None
G. T.	M	52	5.0 to 10.0 mg.	12	590 mg.	Maximal	None	Malignant lymphoma with metastasis to spinal cord	None
R. B.	M	42	3 mg.	4	30 mg.	Maximal	None	Retropituitary undifferentiated malignant neoplasm	None
M. B.	F	62	5 mg.	3	40 mg.	Maximal	None	Multiple sclerosis. Cordotomy done	None

## SUMMARY

1. Nu-2206 has desirable analgesic properties when administered subcutaneously in doses of 3.0 to 6.0 milligrams.
2. For most adults, 1.5 mg. Nu-2206 is an ineffective dose.
3. The duration of analgesia is more prolonged than with 10 to 15 mg. morphine sulfate.
4. Untoward reactions to Nu-2206 are not severe or frequent.
5. The prolonged effect of Nu-2206 is beneficial in those patients who require protracted relief and in whom frequent injections with shorter-acting analgesics are undesirable.
6. No withdrawal symptoms have been observed in this study.

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# THE EFFECT OF SPLEEN PROTECTION ON MORTALITY FOLLOWING X-IRRADIATION

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**L**EAD protection of the surgically mobilized spleen of CF-1\* mice during the delivery of 600 r. total body x-radiation obviates the development of anemia and significantly lessens the severity and duration of the leucopenia and thrombocytopenia that regularly follow total body exposure at this level.<sup>1</sup> On the basis of previous work,<sup>2</sup> a single total body exposure to 550 r. x-radiation produces death of one-half of the mice of this strain in twenty-eight days ( $LD_{50}/28$  days).† Survival data on groups of mice exposed to single dosages of total body radiation with or without lead protection of the surgically mobilized spleen are presented in this paper.

## MATERIAL AND METHODS

The mice used in this study were all females and were 10 to 12 weeks of age when the experiment was initiated. The mice were kept in the animal farm in a constant temperature room (74° F.) for four to six weeks before use and were maintained on a diet consisting of Derwood‡ and water ad libitum before and after the experimental procedures described below.

**Dosimetry.**—The x-rays administered in these experiments were generated in a 250 kv. machine operating at 15 milliamperes. A 0.25 mm. copper filter was used. The half-value

From the Argonne National Laboratory, the Department of Medicine, and the Institute of Radiobiology and Biophysics of the University of Chicago.

Aided in part by a grant from the American Cancer Society upon recommendation of the Committee on Growth and from The Armour Laboratories.

Received for publication, Aug. 3, 1949.

\*CF-1, raised by Carworth Farms. Homozygous for the recessive genes aa bb cc.

†Hereafter referred to as  $LD_{50}$ .

‡Manufactured by the Derwood Mill, Derwood, Md. Consists of skim milk, ground wheat, yeast, corn oil, salt, iron citrate, shark liver oil, Delsterol.

TABLE I. SURVIVAL OF MICE FOLLOWING SINGLE TOTAL BODY X-

GROUP	DOSAGE (r)	SPLEEN SHIELDED	TOTAL NUMBER OF MICE	NUMBER OF SURVIVORS*	PER CENT SURVIVAL
IV	600	Yes	63	54	85.7
III	600	No	75	30	40
IV	700	Yes	27	26	96.3
III	700	No	11	0	0
IV	900	Yes	60	41	68.3
III	900	No	44	3	6.8
IV	975	Yes	23	10	43.4
III	975	No	12	0	0
IV	1,050	Yes	23	7	30.4
III	1,050	No	11	0	0
IV	1,200	Yes	19	0	0
II	0	Operation only	64	64	100
I	0	No operation	54	52	96

\*On Oct. 3, 1949, five or more months after these exposures all mice which had lead protection of the spleen during irradiation still survive and appear grossly normal.



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## EXPERIMENTAL PROCEDURE

As is indicated in Table I, four groups of mice were studied. Mice in Group I were untreated controls. The mice in Groups II, III, and IV were anesthetized with Nembutal (0.072 mg. per gram mouse in a 1:10 dilution) given intraperitoneally, the abdomen was shaved, an incision was made in the left upper quadrant, and the spleen was brought out through the incision. The main pedicle was left intact but to facilitate mobilization a small

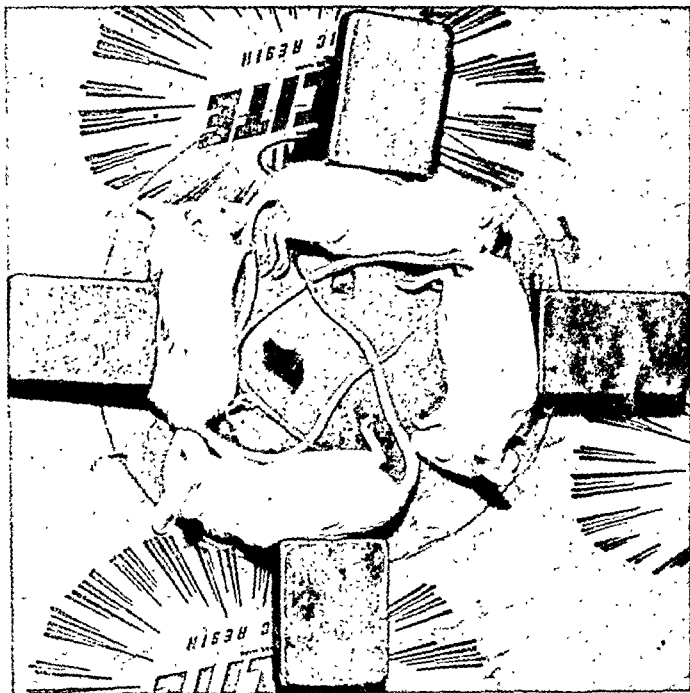


Fig. 3.—Photograph of mice showing surgically mobilized spleens within lead boxes. The mice were irradiated in this arrangement.

vessel at the distal end of the spleen was cut but not tied. Severing this vessel regularly produced an infarct in the tip of the spleen. The infarct varied in size involving up to one-fourth of the distal tip. No significant bleeding was encountered. During irradiation, which required from nine to twenty-two minutes, depending on the dose given, the spleens of Group III mice were placed in paraffin boxes that offered no appreciable shielding (Fig. 1); the spleens of Group IV mice were placed in lead boxes with walls of one-fourth inch thickness (Fig. 2, A and B and Fig. 3) that afforded essentially complete shielding of the spleen from irradiation. The spleens of Group II were kept in lead or paraffin boxes during the x-irradiation.

tion of Groups III and IV. The spleens of all groups thus mobilized were kept moist with physiologic saline during the period in which they were outside the body cavity. After completion of these procedures, the spleens of all groups were returned to the abdominal cavity and the incision was sutured with silk. Complete recovery from the anesthetic required from two to three hours. The mice were returned to the animal farm, and deaths were recorded in twenty-four hour periods through twenty-eight days.

### RESULTS

As indicated in Table I, the percentages of survival of animals in Groups I and II (untreated controls and operated controls) were 96 and 100 per cent respectively. With a dosage of 600 r., 40 per cent of the animals in Group III survived the twenty-eight day period. Accordingly, the  $LD_{50}$  for animals whose spleens were mobilized but not protected was somewhat less than 600 r. and probably close to the  $LD_{50}$  of intact animals (550 r.). On the other hand, in mice of Group IV, which were provided with lead protection of the spleen during irradiation of the balance of the body, a dosage of 975 r. was required to reduce survival to about 50 per cent. In other words, these data show that the  $LD_{50}$  for total body x-irradiation exclusive of the lead-protected spleen is nearly twice as great as the  $LD_{50}$  for total body x-irradiation inclusive of the spleen.

### DISCUSSION

The precise mechanism whereby lead protection of the spleen so significantly increases survival following irradiation is yet to be determined. The capacity of the spleens of these mice to compensate so quickly for the destruction of hematopoietic tissue elsewhere in the body may be the significant factor, but other possible direct or indirect functions of the spleen may be involved. For example, data from a preliminary experiment<sup>3</sup> indicate that rabbits retain the capacity to produce antibodies to intravenously administered foreign red cells (1 c.c. of a 2 per cent suspension of sheep red cells) in a normal manner if either the spleen or appendix is lead protected during the delivery of 800 r. x-radiation to the balance of the body; total body x-irradiation with this dosage but without lead protection of the spleen or appendix more or less completely inhibits the development of demonstrable antibodies to this antigen. The antigen was given two days after irradiation and observations on the development of hemolysin titer followed at intervals of seven days through twenty-eight days.

### SUMMARY

Female CF-1 mice of 10 to 12 weeks of age were divided into four groups. Group I mice were untreated controls. Mice in Groups II, III, and IV were anesthetized with Nembutal, an incision was made in the left upper quadrant of the abdomen, and the spleen was mobilized. Mice in subgroups of Groups III and IV were given various single dosages of x-irradiation. During irradiation the mobilized spleens of Group IV mice were protected from radiation by lead shielding, whereas no shielding was provided for the spleens of mice in Group III. Group II mice served as "operated" controls. The dosages administered were 600, 700, 900, 975, 1,050, and 1,250 r. Irradiation time ranged between

nine and twenty-two minutes. After irradiation the spleen was returned to the abdominal cavity and the incision was sutured. The survival of the mice in these various groups was observed over a twenty-eight day period. Without lead protection of the spleen, the  $LD_{50}$  for irradiated mice in the twenty-eight day period of observation was less than 600 r., whereas the  $LD_{50}$  for mice with lead protection of the spleen approximated 975 r.

#### CONCLUSIONS

The  $LD_{50}$  for mice exposed to total body x-radiation exclusive of the surgically mobilized lead-protected spleen is nearly twice as great as the  $LD_{50}$  for mice exposed to total body x-radiation inclusive of the spleen.

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Similar experiments were carried out on denervated heart-lung preparations.<sup>9, 10, 11</sup> The data from these experiments indicated that as left ventricular output was diminished (e.g., from cardiac damage by asphyxia, "toxic factors," or acute strain), venous engorgement of the lungs occurred, ultimately causing severe edema. The work of Barry<sup>12</sup> was of particular interest. He observed in heart-lung preparations that, if the viscosity of the blood was decreased by the addition of saline, edema of the lungs could be induced. This effect was apparent when the specific gravity of the blood was reduced from 1:053 (normal) to 1:045 or 1:050. However, Barry also observed in these preparations that excessive venous inflow, together with high peripheral arterial resistance, overloaded the left ventricle so that congestion and alveolar edema ensued. Barry's observations were of significance in their demonstration that the Starling principles<sup>13</sup> of vascular pressures and tissue fluid balance may apply to the lung as well as to structures supplied by the peripheral vascular system.

On the basis of such experiments, the concept of pulmonary engorgement and transudation from predominant left ventricular failure has been widely accepted, and much of the rationale of the management of clinical heart failure has rested upon this premise.

Many observers, however, have accepted the alternative concept that pulmonary edema may result from intense reflex vasodilatation of the vascular bed of the lungs or from increased capillary permeability. They have argued that failure of the heart may not be primarily concerned in initiating pulmonary edema. Lambert and Gremels<sup>15</sup> insisted that the formation of edema in heart-lung preparations was accompanied by only a slight rise of pulmonary venous and arterial tensions. These pressures became significantly elevated after edema was established. They thought the capillary leakage was caused by the "toxicity" of the shed blood used in the preparations. A number of others<sup>12, 16, 17, 18</sup> have shared the view that toxic factors occurring in the blood may injure the pulmonary capillaries and facilitate transudation.

Cataldi<sup>20</sup> used a technique similar to that of Coelho and Rocheta,<sup>14</sup> and he noted the precipitation of pulmonary edema when portions of the right ventricle were destroyed by solutions of silver nitrate or absolute alcohol introduced into the ventricular wall. He contended that failure of the left ventricle was not a necessary prerequisite to the onset of pulmonary edema, for damage to the right ventricle might be equally deleterious to the lungs.

The contention that pulmonary vascular reflexes may lead to lung edema formation has rested largely on observations entailing marked disturbances of the sympathetic nervous system.<sup>19, 22-27</sup> Luisada<sup>19</sup> produced paroxysms of edema in rabbits by the use of adrenaline. The paroxysms were often prevented by the use of sedatives (i.e. morphine or the barbiturates), or by transection of the spinal cord at the cervical level. He attributed these effects of adrenaline to stimulation of the central nervous system including centers controlling pulmonary vascular reflexes and capillary permeability. In contrast, evidence has been evolved to show that adrenaline may seriously affect the heart<sup>20</sup> and provoke myocardial failure.<sup>21</sup> Luisada and Sarnoff<sup>22</sup> declared that experimental

procedures producing cerebral anemia may alter the distribution of blood within the lungs by vasomotor mechanisms, so that alveolar transudation occurs. A similar explanation was suggested by Jarisch and co-workers<sup>23</sup> for the edema resulting from the intracisternal injection of veratrine in rabbits. Farber<sup>24</sup> reported that edema of the lungs frequently occurred in guinea pigs following bilateral cervical vagotomy causing the loss of vasomotor control of the lungs. However, Sussman and co-workers<sup>25</sup> could find no such effect of vagotomy in guinea pigs when respiration was optimally maintained.

On the basis of clinical observation, a number of workers have suggested that pulmonary edema may be touched off by failure of autonomic control of the pulmonary vascular bed. Salmon<sup>30</sup> implicated stimulation of the carotid sinus as a means of inducing transudation. Intracranial hemorrhage<sup>31</sup> and injuries of the spinal cord<sup>32</sup> and of the medulla oblongata<sup>33</sup> have likewise been reported to cause intense pulmonary edema.

In most of the clinical and experimental observations of "neurogenic" pulmonary edema, measurements of cardiac performance were not reported. If the function of the heart were seriously curtailed through aberrant autonomic stimulation from various causes, one might expect lung congestion and its consequences from a critical decrease of left ventricular output. In fact, Campbell, Haddy, and Visscher<sup>34</sup> have shown recently that elevation of intracranial tension may be accompanied by marked bradycardia, diminution of cardiac output, and elevation of pulmonary venous and arterial pressures, with the occurrence of pulmonary edema. These responses were modified by severance of the vagus nerves.

The conflicting views of the genesis of pulmonary edema, as indicated in this review, have not been reconciled. Luisada has stated that "the generally unsatisfactory basis for the backward failure theory of pulmonary edema strongly favors its abandonment."<sup>13</sup> On the other hand, there is a paucity of evidence to support the "neurogenic theory" as the sole cause of edema of the lungs. Furthermore, other evidence has indicated that the latter concept must be viewed with skepticism until reflex effects upon the heart and its function have been investigated more thoroughly.

It seems clear however that any attempt to re-explore the cause of pulmonary edema requires an understanding of the means by which abnormal fluid exchange may occur in the lungs. Although the concise precepts of fluid balance described by Starling<sup>13</sup> are widely accepted as applying to the lung as well as to other tissues, there has been little controlled observation of the Starling principles on the lungs. Experiments were therefore designed to test the effects of increased capillary hydrostatic pressure and of diminished osmotic tension (the Starling principles) on pulmonary circulatory function. Experiments dealing with this aspect of the problem will be reported in this paper.

#### METHOD

Experiments were performed on dogs under Nembutal anesthesia. The right thoracic duct was isolated, and the rate of lymph flow was measured by the method previously described by us.<sup>35</sup> Under positive pressure respiration, the thorax was opened and the

pericardium incised. The animal was then heparinized in the usual manner, after careful hemostasis had been secured. Systemic and pulmonary arterial pressures were recorded from cannulae placed in the left subclavian artery and right upper pulmonary lobar artery; these were connected to simple mercury manometers. A water manometer was attached to a cannula tied into the left atrial appendage. Ordinary heart-lung preparations were then set up.

In order to study the effects of decreased osmotic pressure, the protein content of the blood was reduced by partial replacement of the plasma with Locke's solution (six experiments). Dilution was accomplished by centrifuging a quantity of blood approximately equal to that in the circuit; the plasma was removed to the level of the packed cells by suction, and the original plasma volume was then restored by the addition of warmed Locke's mixture. At any given time, the normal blood in the venous reservoir could be drained away and replaced by the red cell suspension. The rates of lymph flow, the various pressures, and the appearance of the lungs were repeatedly observed. Sections of the lungs for histologic examination were taken during and at the termination of the experiments. Plasma protein levels were determined by the methods of Campbell and Hanna<sup>36, a</sup> and of Howe.<sup>36, b</sup>

The effect of increased pressure in the pulmonary capillaries on edema formation was studied in six preparations. Pulmonary engorgement was produced by "overloading" the left ventricle. Elevation of venous inflow (and therefore cardiac output) or increase of the peripheral resistance, singly or together, produced left ventricular dilatation and left atrial engorgement. The effects of this treatment on pulmonary arterial and venous pressures, and on blood volume, were noted. Lymph flow was frequently determined, and sections for microscopic study of the lungs were taken.

All specimens of lung tissue were fixed in formol-saline solution (Trowell<sup>37</sup> so as to facilitate preservation of edema fluid and were stored in 80 per cent alcohol.

## RESULTS

*Effects of Diminution of Plasma Proteins on the Formation of Pulmonary Edema.*—During the control periods, the heart-lung preparations functioned under loads that appeared to place no strain upon the myocardium. Usually the peripheral resistance was adjusted to produce a mean aortic pressure of 90 to 110 mm. Hg; the cardiac inflow was about 400 c.c. per minute. Under these conditions, mean pulmonary arterial pressure varied from 10 to 20 mm. Hg and left intra-atrial tension ranged from 3 to 6 cm. of water. Thereafter the suspension of cells in Locke's fluid was placed in the venous reservoir after removal of as much of the normal blood from the circuit as possible without introduction of air into the venous tubing. Usually 200 to 300 c.c. of the cell suspension were used. In most experiments, it was estimated that approximately one-half of the circulating blood volume was replaced when these quantities of cell suspension were used.

The effects of the addition of diluted blood were first manifested in the flow of lymph from the lungs. The flow increased notably within one to ten minutes, and thereafter was progressively accelerated. The level of blood in the venous reservoir decreased, indicating a loss of circulating blood volume. However, the size of the heart was not increased, and pressures in the pulmonary arteries and left atrium were not materially altered. In four experiments, pulmonary arterial pressure was not affected at all; in one instance a decrease of 4 mm. Hg tension was noted, while in two examples there were transient elevations of the pulmonary blood pressure of 5 mm. Hg. Pulmonary venous pressures were unaltered in two instances, and rose from 2 to 6 cm. of water in four

preparations. Therefore, the continued normal size of the heart and the general level of performance indicated that cardiac function was not impaired (Fig. 1 and Table I).

After the cell-saline suspension was circulating in the preparations, and during the period of fluid loss into the lungs, there was a gradual return of plasma protein concentration to normal values. In some instances the plasma

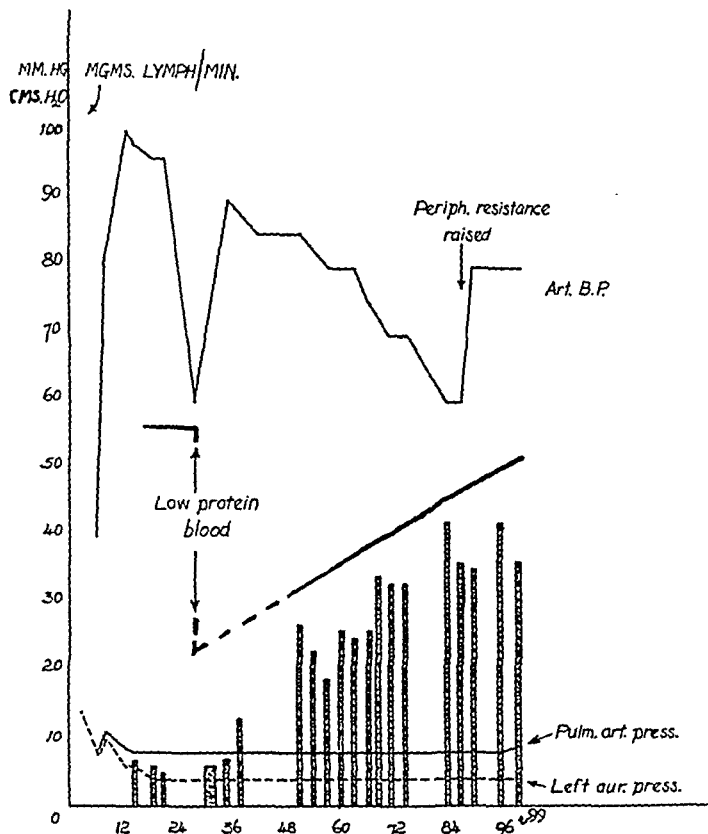


Fig. 1.—The effect of hypoproteinemia upon fluid exchange in the lungs. At the time indicated, low protein blood was placed in the heart-lung system. Protein concentration of the blood is indicated by curve ————. Columns represent lymph flow. Abscissa is time in minutes. See Table I, Experiment 48.

proteins continued to rise to concentrations greater than those in the normal, undiluted blood. (See Table I.) However, with the rise of plasma protein concentrations to control (or above control) values, a corresponding diminution of lymph flow and reduction of pulmonary edema was *not* observed. This phenomenon is well illustrated in Fig. 1. Note that the curve indicating plasma protein concentration of the cell suspension showed a progressive increase, but that lymph flow also showed a continuous rise. The persistence of accelerated lymph flow after the return of plasma proteins to normal levels probably represented the drainage of the previously accumulated fluid from the lungs. However, the mechanism by which the plasma proteins subsequently rose to levels

TABLE I. EFFECT OF DIMINUTION OF PLASMA PROTEINS

EXPERIMENT	CHANGE IN		LYMPH FLOW		PLASMA PROTEIN			PULMONARY EDEMA
	PULMONARY ARTERIAL PRESSURE (MM./HG)	LEFT AURICULAR PRESSURE (CM./H <sub>2</sub> O)	CONTROL (MG./MIN.)	AFTER DILUTION (MG./MIN.)	CONTROL (GM. %)	DILUTED (GM. %)	FINAL (GM. %)	
44	+5 to +2	+5 to +3	40	150		3.1	7.0	Present
48	0	0	5	42	5.5	3.1	5.8	Present
49	+5 to 0	+2	6	16	5.2	2.3		Present
51	0	+4	16	78	6.5	2.1	8.5	
53	0	+6			5.5		9.8	Present
54	0	0	98	150	7.4		8.2	Present
			Av.	Av.				

above control values is not clear. Possibly the interstitial fluid produced by the initial hypoproteinemia obstructed aeration of the lung tissue, causing anoxic damage to the pulmonary capillaries and abetting transudation of protein-poor fluid. Another explanation may be that plasma protein lost into the extravascular space during initial transudation increased tissue fluid osmotic tension to such a degree that transudation went on despite the increase of blood protein level, producing greater hemoconcentration.

*Influence of Cardiac Insufficiency and Pulmonary Congestion on the Formation of Edema of the Lungs.*—These observations were carried out on six heart-lung preparations. For control purposes, the venous inflow and peripheral resistance were adjusted so as to impose no undue strain upon the hearts, as in the preceding experiments. In four experiments the cardiac load was augmented by increasing the aortic pressure from 90 to 110 mm. Hg to 160 to 180 mm. Hg. In two instances, overburdening of the heart was accomplished by sharp elevations of venous inflow to 600 to 700 c.c. per minute (control, 200 to 300 c.c. per minute). With both means of taxation of the heart, left ventricular dilatation became apparent. The pulmonary venous and arterial pressures then rose and congestion promptly ensued. Congestion of the lungs was manifested by turgescence, followed quickly by duskiness of the tissue. Myriads of fine crepitant râles could be discerned by direct auscultation. The fluid volume in the venous reservoir diminished rapidly, and the passage of lymph from the right thoracic duct progressively increased beginning three to twelve minutes after the onset of congestion. As the process continued, the pulmonary lymph became pinkish in hue, and finally dark red. Microscopic examination of the red lymphatic fluid showed that it contained up to 600,000 red blood cells per cubic millimeter. Sections of the lung tissue taken at the time of accelerated lymph flow exhibited intense congestion and edema. In some instances pulmonary transudation was so marked that foam extended into the main bronchi and trachea. In all of these experiments, pulmonary engorgement and edema was permitted to become extreme; no attempt was made to reverse the process by reducing cardiac load.

The difference in appearance between these lungs and those of the hypoproteinemic experiments was striking. When the hypoproteinemic lungs became edematous, they were pale and flaccid, the acceleration of lymph flow was not so rapid, and the general tempo of the reaction was rather gradual. In contrast, the congested lungs became red and turgid, the acceleration of lymph flow was rapid, and the lymph became hemorrhagic. Edema fluid was frequently profuse.

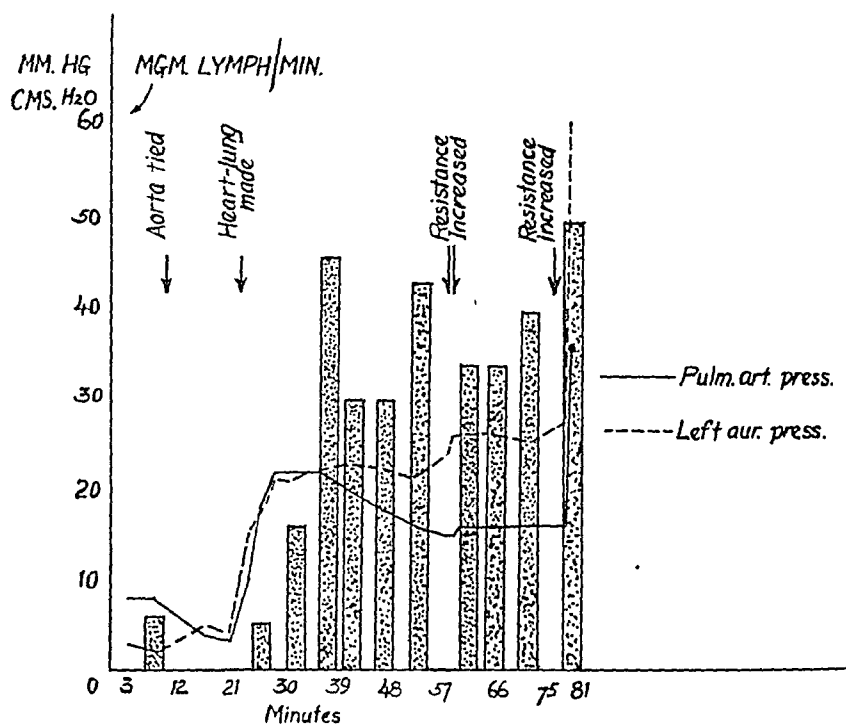


Fig. 2.—The effects of pulmonary congestion upon fluid exchange in the lungs. When the heart-lung was established in this experiment, peripheral resistance was excessive and pulmonary congestion immediately appeared, as indicated by venous and arterial pressure curves. Congestion was further increased at arrows. Columns represent lymph flow.

#### DISCUSSION

These experimental data suggest that the well-known Starling principles of vascular and tissue fluid balance are operative in the lung structures. The mechanism of pulmonary edema induced by low plasma protein—low blood osmotic tension—is clear and requires no particular comment. However, the production of pulmonary edema from congestion is more important from the standpoint of cardiac insufficiency.

The experiments show that in the heart-lung preparation, left ventricular load may be greatly augmented by elevation of aortic pressure or by increasing the stroke and minute output or by both. Thus the left ventricle can be overloaded so that venous inflow (and right ventricular output) may exceed the greatest possible left ventricular output. With failure of the left ventricle to accept all of the blood flowing to it through the lungs, pulmonary venous

outflow is impaired and blood is impounded in the lungs by the continuing competence of the right ventricle. Examination of Fig. 2 and Table II shows that these events cause pulmonary venous and arterial pressures to rise to high levels. Since the normal pulmonary capillary pressure is estimated at mean values of 10 to 15 mm. Hg in open-chest preparations<sup>28</sup> and the normal blood osmotic pressure at 20 mm. Hg (judged by normal plasma protein levels in these and other<sup>29</sup> experiments), it is apparent that capillary pressure may exceed that of the osmotic tension when the lungs are congested. Rapid filtration of fluid from the capillaries then occurs.

TABLE II. EFFECTS OF PULMONARY CONGESTION

EXPERIMENT	RISE IN		LYMPH FLOW		PULMONARY EDEMA	CARDIAC OUTPUT (C.C./MIN.)
	PULMONARY ARTERIAL PRESSURE (MM./HG)	LEFT AURICULAR PRESSURE (CM./H <sub>2</sub> O)	CONTROL (MG./MIN.)	AFTER CONGESTION (MG./MIN.)		
35		59	65	145	Present	
38	36	57	6	63	Present	
39	48	74	40	185	Present	
40	50	84			Present	
41	34	50			Present	500 to 680
43	5	14			Present	160 to 810

A contrasting explanation for experimental pulmonary edema was offered by Luisada and Sarnoff.<sup>22</sup> They reported experiments in which saline infusions, given in large quantity, produced pulmonary edema. They noted that when the solutions were injected cephalad into the carotid arteries under high pressure, edema of the lungs frequently occurred. On the other hand, when comparable amounts of fluid were injected into the systemic veins under lower pressure, edema was somewhat less frequent. They suggested that forcible irrigation of the cerebral circulation by salt solutions resulted in cerebral anemia and provoked pulmonary transudation by reflexogenic influences on the vascular bed of the lungs. Their results were compatible with sudden and serious cardiac overload. They paid scant attention to the possibility of altered circulatory dynamics, and the influence of massive saline infusions on the plasma proteins was not considered.

Other workers<sup>40, 41</sup> have also reported that massive increases in the circulating fluid volume may introduce a load against which the heart cannot cope, with consequent pulmonary congestion and edema. This factor was particularly striking in the studies of Gibbon and Gibbon.<sup>27</sup> In cats, they removed the right middle and both lower lung lobes so as to raise the pulmonary hydrostatic pressure by restriction of the circulatory capacity of the lungs. They found that intravenous saline infusions were especially likely to provoke pulmonary edema and rapid death in these preparations. Although these workers attributed the edema mainly to increased capillary permeability rather than to any dynamic effects, their results suggest that edema occurred from elevation of the pulmonary hydrostatic pressure and possibly from diminution of the plasma proteins.



The correlation of increased lymph flow and the onset and progression of pulmonary edema was strikingly evident in these experiments. We noted that in every instance where a significant elevation of lymph flow occurred, gross or microscopic pulmonary edema could be found.

#### SUMMARY

Experiments are presented which show that pulmonary edema may be induced in the heart-lung preparation by (1) the lowering of the plasma proteins from replacement of blood plasma with Locke's solution and (2) by elevation of pulmonary vascular (hydrostatic pressures following imposition of left ventricular overload. It was demonstrated that abnormal fluid exchange in the lung tissue may result from disturbances of hydrostatic and osmotic pressure relationships as described by Starling.<sup>13</sup> Although the results of these experiments, involving the heart-lung preparation, do not necessarily explain the cause of acute pulmonary edema in intact subjects, they do demonstrate that Starling's principles apply to the pulmonary as well as to the systemic circulation. The Starling principles, therefore, must be considered in all future studies relating to the pathogenesis of pulmonary edema.

The onset and progression of pulmonary edema were always attended by an increase in the flow of lymph from the right thoracic duct. The measurement of an increased pulmonary lymph flow has been found to be a reliable indicator of the presence of pulmonary edema.

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## LABORATORY METHODS

### POLYVINYL ALCOHOL-FIXATIVE AS A PRESERVATIVE AND ADHESIVE FOR PROTOZOA IN DYSENTERIC STOOLS AND OTHER LIQUID MATERIALS

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TROPHOZOITES of intestinal amoebae deteriorate rapidly, and consequently it is frequently difficult for a physician to get laboratory confirmation of suspected cases of amoebic dysentery or amoebiasis. Submitting the stool to a distant laboratory for diagnosis has been unsatisfactory since the etiological agent, even if originally present, is usually unrecognizable by the time the specimen is examined. A negative report under these circumstances is, of course, meaningless.

In an attempt to solve this problem, Goldman<sup>1, 2</sup> devised a technique for preserving fecal smears with a film of polyvinyl alcohol (PVA)-fixative. Smears so prepared could be shipped for long distances or stored in a dried condition for months without undergoing any deterioration. In the laboratory, the PVA films were removed and the smears were stained by standard hematoxylin procedures.

During 1948, this method was used by this laboratory in cooperation with local health officials and physicians in Georgia, Alabama, Mississippi, Kansas, North Dakota, Minnesota, and California. Out of thirty specimens submitted during several months, a diagnosis of *Endamoeba histolytica* was made in thirteen instances. The rest showed either no organisms or trophozoites and cysts of various other intestinal protozoa (*Endamoeba coli*, eleven; *Endolimax nana*, fifteen; *Dientamoeba fragilis*, one; and *Giardia lamblia*, one).

The purpose of the present paper is to report simplified and improved methods for preparing specimens and for handling them in the laboratory, and to point out possible applications of the technique to the study of organisms other than intestinal protozoa.

#### MATERIALS AND METHODS

*Polyvinyl Alcohol.*—Polyvinyl alcohol is a synthetic water-soluble polymer of vinyl alcohol that is available in the form of a white, odorless powder or granular material. Its aqueous solutions are stable over long periods of time. Viscosity of the solutions can be regulated

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by concentration of PVA and by the degree of polymerization of the alcohol.\* Films formed by drying aqueous solutions are tough, thin, and transparent. They generally adhere firmly to clean glass surfaces and are resistant to alcohol, ether, acetone, xylol, oils, and even short exposures to water.

PVA has been used as an embedding medium for tissues,<sup>3</sup> as a mounting medium for insects and fungi,<sup>4, 5, 6</sup> and as a means of reducing motility of paramecia and other small organisms.<sup>7</sup>

*PVA-Fixative.*—The fixative was prepared as described by Goldman.<sup>2</sup> Five grams of powdered PVA were added to a mixture at room temperature containing 1.5 ml. of glycerol, 5 ml. of glacial acetic acid, and 93.5 ml. of Schaudinn's solution (2 parts of saturated aqueous mercuric chloride to 1 part of 95 per cent ethyl alcohol). Heating to approximately 75° C. while stirring facilitated the preparation of a water-clear, nonlumpy solution. This was used as soon as it had cooled to about 50° C. or less and it remained satisfactory for several months.

*Preservation of Specimens With PVA-Fixative.*—

*A. On Microscope Slides:* With the aid of an applicator stick, a drop of specimen was mixed on a microscope slide with 3 drops of fixative and smeared over approximately one-third of the glass surface. To insure thorough drying, smears were usually placed in an incubator at 37° C. overnight.

*B. In Vials:* A quantity of specimen was thoroughly mixed in a vial containing three or more parts of fixative. Smears for staining were prepared immediately or months later by spreading a drop or two of the mixture on a microscope slide and allowing it to dry thoroughly. Care was taken not to have the smears too thick.

*Staining of Organisms in PVA Films.*—In the original PVA-fixative technique, fecal smears were covered but not mixed with fixative. In the laboratory the films were removed before staining the smears. The present methods of preservation were made practical by the observation that PVA films are permeable to all commonly employed staining reagents. This makes it possible to stain dried PVA smears in the same manner as smears fixed by conventional methods with Schaudinn's fixative.

Inasmuch as critically stained organisms were desired, the long Heidenhain iron-hematoxylin procedure usually was employed. Diagnostically satisfactory preparations were obtained by more rapid staining procedures such as the ones proposed by Tompkins and Miller<sup>8</sup> and Goldman.<sup>9</sup> Delafield's hematoxylin was used in some instances, as in staining *Paramecium*. In all of the staining procedures, dried films were first placed in 70 per cent alcohol containing iodine for ten minutes or longer in order to remove mercuric chloride crystals.

Ordinarily, if the PVA film on a slide is thoroughly dried before being stained it adheres perfectly. In rare instances, for reasons not entirely understood, films may wrinkle around the edge or in the middle and show a tendency to slip off the slide during prolonged exposure to aqueous solutions. This tendency may be minimized by preparing relatively thin, rectangular smears. To prevent the loss of a wrinkling film, the slide may be placed in the incubator in a horizontal position and allowed to dry before continuation of the staining process.

In order to test the effect of drying organisms in PVA films, such films were thoroughly dried (over three hours at 37° C.) after each of the different steps in the iron-hematoxylin procedure. After each interruption, the slides were placed in the next solution and the staining was completed in the usual manner. Only those slides were unsatisfactory which were dried after removal from the graded alcohols during dehydration. Therefore, contrary to the usual direction never to allow fecal smears to dry during the staining procedure, in the PVA-fixative technique no particular harm is done at most of the steps.

\*Polyvinyl alcohol is marketed under the trade name of Elvanol in various grades representing degrees of polymerization and hydrolysis. The product used in this study was Elvanol 90-25 obtained from E. I. du Pont de Nemours and Co., Electrochemicals Department, Wilmington 98, Del. This does not represent an endorsement of the product by the Public Health Service.

## OBSERVATIONS AND DISCUSSION

No differences have been observed between organisms stained after preservation with PVA-fixative and those stained after usual methods of Schaudinn fixation. Nuclear details and cytoplasmic inclusions are clearly defined. Rapid and complete fixation apparently occurs, since many trophozoites exhibit protruding pseudopods.

One distinct advantage of PVA-fixative is that it makes possible successful staining of organisms in liquid specimens. Anyone who has ever attempted to prepare permanent stained mounts of protozoa in cultures, watery stools, or pond water has experienced the difficulty of retaining organisms on the slide during staining procedures. When PVA-fixative is used, it serves to adhere as well as to preserve the organisms and prevents their loss during staining. Stained slides containing many organisms have been made from the following liquid specimens: diarrheic stools and liquid overlay of cultures containing trophozoites of *E. histolytica* (Figs. 1 and 2); loose stools containing trophozoites of *E. coli* (Fig. 3), *Iodamoeba buetschlii* (Fig. 4), *E. nana* (Fig. 5), *D. fragilis* (Fig. 6), and *G. lamblia* (Fig. 7); cultures and pond water containing *Paramecium* (Fig. 8) and other protozoa; and tap water containing hatched miracidia of *Schistosoma mansoni* (Fig. 9). The only other way known to the authors to stain consistently large numbers of organisms in such specimens is the bulk centrifuge method, employing test tubes. However, the PVA-fixative technique is considerably simpler, particularly when only a few slides are to be prepared.

The technique is also useful for preserving interesting specimens or teaching materials for subsequent staining. A variety of organisms have been preserved in liquid fixative and in dried films for about a year without showing any changes when stained. Of particular interest is a specimen of aspirated material from an amoebic lung abscess preserved in a vial with PVA-fixative (Fig. 10).

At present PVA-fixative cannot be recommended unreservedly for preserving specimens containing cysts. Larger cysts are frequently distorted, apparently during drying of the PVA film. However, in specimens containing many cysts, enough of them with distinct characteristics are usually present to insure correct identification. Occasionally, exceptionally good preparations are obtained even of *E. coli* (Fig. 11) and *I. buetschlii* (Fig. 12) cysts. Smaller cysts such as *G. lamblia* (Fig. 13) and *E. nana* (Fig. 14) are usually not adversely affected. As would be expected, staining PVA-fixative films is not an efficient technique for diagnosing helminth eggs.

In general, stool specimens containing cysts and eggs are best examined in wet mounts prepared either directly from the specimens or following a concentration procedure. In such mounts the refractive appearance of unstained cysts and eggs makes them relatively easy to detect with a low-power objective. Helminth eggs and some cysts usually can be diagnosed in temporary mounts of PVA-fixed specimens, but the fixative destroys the refractiveness of the organisms and renders them more difficult to find and identify.

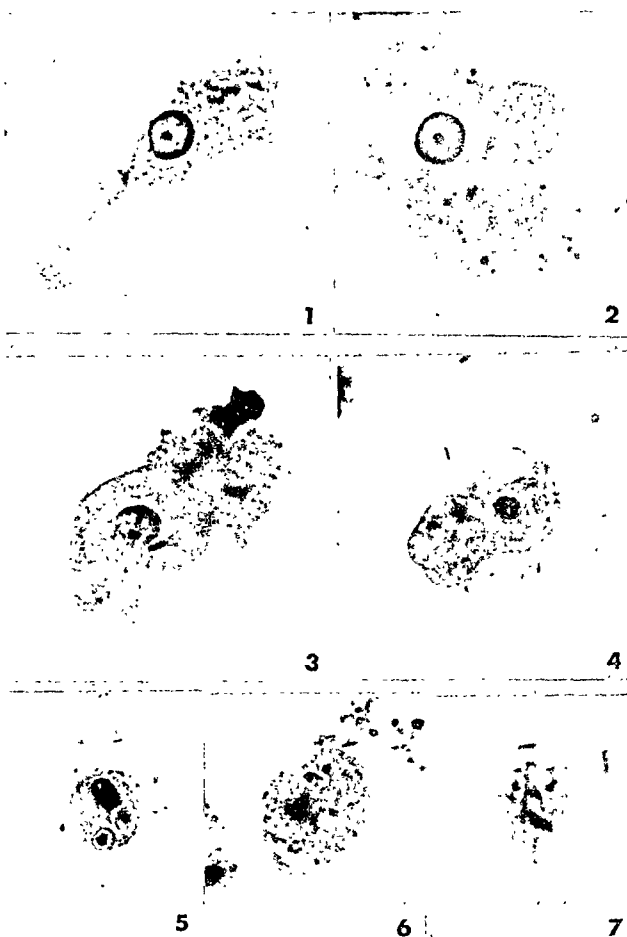


Fig. 1.—*E. histolytica* trophozoite from diarrheic stool. Note protruding pseudopodia.

Fig. 2.—*E. histolytica* trophozoite from liquid overlay of a culture

Fig. 3.—*E. coli* trophozoite from loose stool

Fig. 4.—*I. buetschlii* trophozoite from loose stool.

Fig. 5.—*E. nana* trophozoite from loose stool

Fig. 6.—*D. fragilis* trophozoite from loose stool

Fig. 7.—*G. lamblia* trophozoite from loose stool.

(All photographs are of organisms stained in PVA-fixative films. Except as indicated films were stained by the long Heidenhain iron-hematoxylin procedure and were photographed at a magnification of 1,125 diameters.)

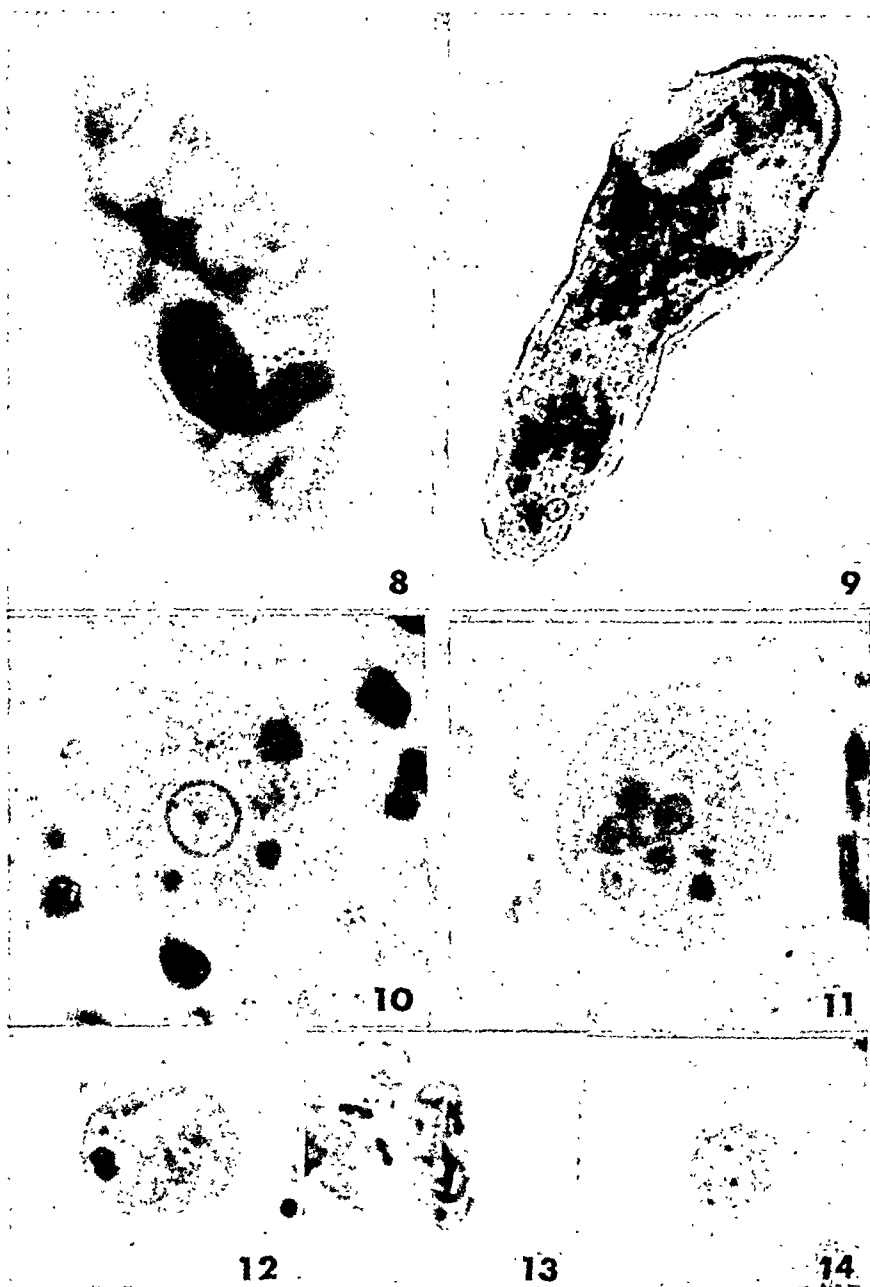


Fig. 8.—*Paramecium* sp. from hay infusion culture. Note micronuclei. (Delafield's hematoxylin,  $\times 500$ .)

Fig. 9.—*S. mansoni* miracidium from tap water. (Iron-hematoxylin [Goldman, 1949].  $\times 500$ .)

Fig. 10.—*E. histolytica* trophozoite from lung abscess.

Fig. 11.—*E. coli* cysts from stool. Weigert's copper chrome procedure.

Fig. 12.—*I. buetschlii* cyst from stool.

Fig. 13.—*G. lamblia* cyst from stool.

Fig. 14.—*E. nana* cyst from stool.

(All photographs are of organisms stained in PVA-fixative films. Except as indicated films were stained by the long Heidenhain iron-hematoxylin procedure and were photographed at a magnification of 1,125 diameters.)

Although the PVA-fixative technique might be applied to the study of many different types of organisms, it has been developed primarily to assist the public health laboratory in securing diarrheic, dysenteric, purged, and proctoscopic specimens in a satisfactory condition for diagnosis of amoebiasis. The methods described in this report can be adapted easily to this need. For submitting stool specimens, small vials (15 ml. capacity), two-thirds full of PVA-fixative, can be distributed to physicians in the same manner that vials containing other preservatives are supplied. Since the PVA-fixative technique is not a good method for diagnosing cysts and eggs, the physician should also be supplied with a vial containing no preservative. He should be instructed to place approximately 4 ml. portions of *fresh* liquid or formed stool in each vial and to mix the specimen thoroughly in the vial containing fixative.

With the two-vial method of submitting specimens, a variety of parasitological techniques can be performed and demonstrations of protozoa and helminths can be made regardless of their stage of development. For example, the vial containing PVA-fixative will furnish material for many smears which can be stained and examined for trophozoites and cysts of amoebae and other intestinal protozoa. The vial containing no preservative will furnish material for such gross examinations as consistency of stool, blood, mucus, portions of adult worms, etc.; for microscopic examination of temporary mounts (saline and iodine) for protozoan cysts and helminth eggs and larvae; and for concentration procedures for cysts and eggs.

The expense involved in preparing PVA-fixative is nominal. One pound of PVA costs less than a dollar and is sufficient to make enough fixative to fill a thousand 15 ml. vials approximately two-thirds full. Other ingredients of the fixative—mercuric chloride, ethyl alcohol, acetic acid, and glycerine—are standard laboratory reagents.

#### SUMMARY

1. Methods are described for the use of polyvinyl alcohol (PVA)-fixative as a preservative for intestinal protozoa and other parasitic and free-living organisms.

2. Organisms so preserved will remain suitable for staining for several months. Best results are obtained with amoebic trophozoites which are otherwise difficult to preserve.

3. In addition to its preservative action, PVA-fixative acts as an adhesive to prevent loss of organisms from smears during staining procedures.

4. The technique has been developed primarily to aid in the diagnosis of amoebiasis and is readily adapted to this need. A two-vial method for shipping stool specimens is suggested which would enable the laboratory to recover all diagnostic stages of the intestinal parasites.

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## DESIGN OF A PUMP SUITABLE FOR BLOOD

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THERE has been a need in experimental medicine and therapeutics for a pump that could convey blood under sterile conditions without hemolysis, acceleration of the clotting process, or introduction of pyrogens. Our special interest has been in a pump which will bring the blood of a human subject from a vein to a dialysis apparatus for the removal of products of uremia at variable and appropriate rates. Recently, a number of types of extracorporeal blood dialyzers have been made, some of which utilize pumps of various designs. However, none of these completely satisfies all of the above-mentioned requirements. The various dialyzing procedures for the treatment of uremia, in relation to dietary and other measures, have been the subject of a comprehensive review by Snapper.<sup>1</sup> De Leeuw and Blaustein<sup>2</sup> and Vanatta, Muirhead, and Grollman<sup>3</sup> have noted that the Beck pump, which forms an integral part of the original Kolff "artificial kidney," and serves to return blood from the dialyzer to the vein of the patient, itself produces hemolysis of the blood circulating through it. Vanatta and co-workers have eliminated, therefore, the use of the pump for this purpose and were able to substitute gravity for the return of the blood.

In the following report a simple pump will be described that meets our stringent requirements rather well. In this apparatus there are no valves, close-fitting, or metal parts in contact with the blood. Dead spaces where the current moves slowly are eliminated and air bubbles are not introduced. A self-regulating pressure principle provides safety for the cellophane tubing in the dialysis apparatus proper, and sterility is accomplished by autoclaving.

In principle the pump consists of a Tygon tube of suitable wall thickness and diameter which is compressed in a wave-like motion by a series of twelve keys that push the column of fluid ahead. Behind the crest of the wave, the plastic tubing expands by its own resiliency to fill up again. The baffle plate against which the tubing is compressed is adjusted so that the compression of the tubing is incomplete. Pumping will then cease at a predetermined pressure if increased resistance in the outer circuit causes that pressure to be attained. Furthermore, the compression of the tubing is largely performed by one elevated edge of the key rather than by a flat surface, reducing thereby the area in which mechanical trauma may occur.

In the apparatus (Fig. 1) a constant-speed electric motor with a gear device drives a screw-shaped series of twelve rings on an axle. The rings elevate a series of twelve keys to create pressure on the tubing. The rings are evenly spaced at intervals to form one complete sine wave. The amplitude of the wave can be limited by restricting the extent of motion of the keys. The

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tubing lies between the keys and the adjustable plastic baffle. In usage, the tubing is autoclaved and placed on the keys and the baffle is fastened on top. The baffle can be set for complete compression of the tubing, with the attainment of pressures well over 300 mm. Hg. By loosening the set screws, the baffle can be adjusted to allow leakage at any intermediate pressure.

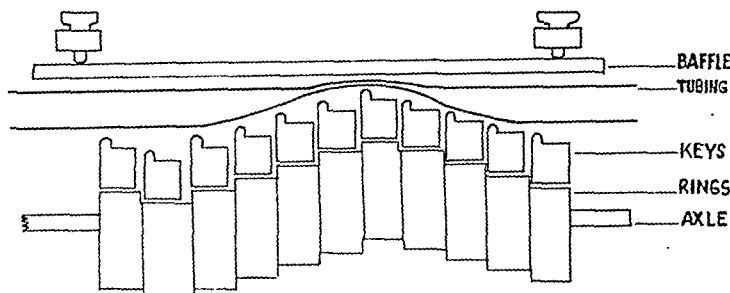


Fig. 1.—Cross-section diagram of pump. Compression incomplete at height of wave.

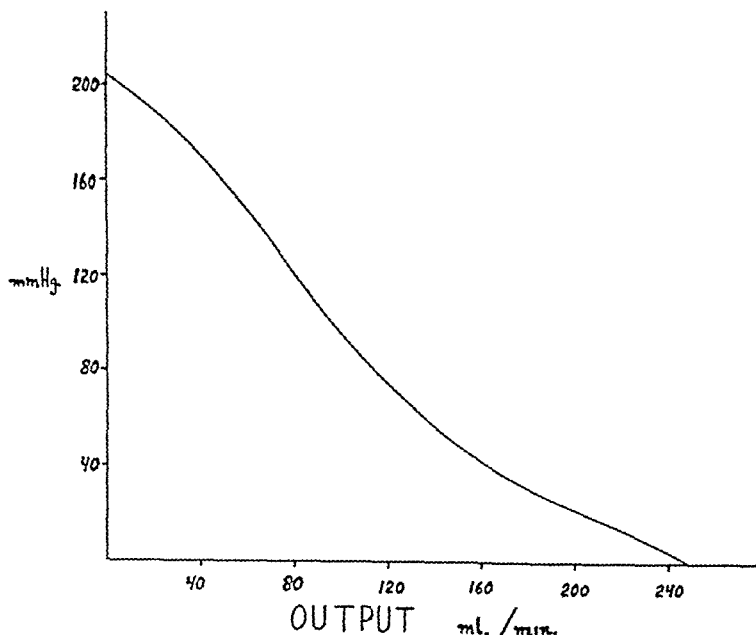


Fig. 2.—Relationship between output and pressure within the system.

In Fig. 2 the inverse relationship between output and pressure within the system is shown in an experiment in which the baffle was regulated to have pumping stop at about 200 mm. Hg. The increase in pressure within the output or arterial side of the system is attained by increased peripheral resistance. Increased resistance on the suction or venous side of the pump would have the same effect of lowering the output of the pump. By limiting the maximal pressure, the cellophane tubing used in dialysis can be safely guarded against undue stress which can result in breakage.

Outputs of from 1 to 18 liters per hour were obtained by varying the stroke volume and the diameter of the tubing used in the model at hand. The output remained constant as long as the peripheral resistance was unchanged.

The pump in the Kolff "artificial kidney" has rollers that create shearing stresses in the tubing and provide a large area for mechanical trauma. In the apparatus described in this report no shearing motion is possible since the keys can move only in the vertical plane. The rate of hemolysis of the blood recirculated through a closed circle of tubing of 20 ml.<sup>3</sup> volume at 37° C. was determined with the pump herein described. Since hemolysis was not observed with normal blood, freshly drawn heparinized blood from a patient in uremia (urea N 148 mg. per cent) was taken for testing. The rate of blood flow was 125 ml. per minute and the maximum pressure attainable was set at 220 mm. Hg. Absolutely no hemolysis was discernible after 12 minutes (72 circuits of the blood). In 18 minutes of recirculation of the blood (108 circuits) a very faint trace and in 24 minutes (144 circuits) a faint trace of hemolysis appeared. Since about 50 circuits of the blood volume through our pump and dialyzer is the maximum recirculation to be expected, the mechanical trauma to the blood by the pump is negligible.

In summary, a pump is described that conveys blood under sterile precautions without hemolysis for 72 circuits of the blood volume. It is without valves, contains no dead space, and provides safety when coupled to a cellophane tube dialyzer by ceasing to pump at predetermined pressures

The cooperation of Mr. G. J. Frank, Jr., and Mr. J. C. Marsh of the Marsh Laboratory, Pittsburgh, Pa., is gratefully acknowledged.

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TABLE I. PREOPERATIVE AND POSTOPERATIVE CULTURES, INTESTINAL FLORA, AND COLIFORM COUNTS

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	<i>Escherichia coli</i>	+				0				0		
	<i>Aerobacter aerogenes</i>	+				+				0		
	<i>Bacillus pyocyaneus</i>	+				+				0		
Preoperative day		10	9	8	7	6	5	4	3	2	1	
Coliform count/gram of feces		10,000								0	0	0
Case 2 (Patient W. O.)	<i>Staphylococcus albus</i>	+				+				0		
	<i>Alpha hemolytic streptococcus</i>	+				0				0		
	<i>Alpha hemolytic streptococcus</i> (enterococcus type)	+				+				0		
	<i>Bacillus pyocyaneus</i>	+				+				0		
	<i>Escherichia coli</i>	+				0				0		
	<i>Proteus vulgaris</i>	+				0				0		
	<i>Bacillus mucosus capsulatus</i>	+				0				0		
	<i>Monilia albicans</i>					0				+		
Preoperative day		13	12	10	9	8	7	6	3	2	0	
Coliform count/gram of feces		10,000	12,000					30,000	5,500	0		

tinal antiseptic therapy. Armed with this information in advance, it should be possible to give systemic or topical treatment with greater speed and specificity for an infection which might develop from the colon after operation. Because of the rapid development of resistance of certain organisms to streptomycin, it was decided to refrain from using it as an intestinal antiseptic but to reserve it for later use in case infection developed.

Cultures of colon contents were obtained through the distal limb of the colostomy. A sterile rubber tube was inserted as far into the bowel as possible and a long slender pipette passed through this tube was used to aspirate colonic juice. All cultures were planted on blood agar plates and inoculated into 6.0 c.c. of beef heart infusion broth enriched with sheeps' blood and fortified with 5.0 mg. per cent of para-aminobenzoic acid, each inoculation consisting of a 2 mm. platinum wire loopful of the juice. Tubes of chopped meat media fortified with infusion broth and sealed with petroleum jelly and paraffin were used for anaerobic cultures. Coliform counts were done by a modification of the streak method described by Poth.<sup>1</sup>

Cultures of feces were taken on the day the colostomies were opened and are shown in Table I. Colostomy and rectal enemas and instillations of 25 per cent magnesium sulfate served to clean out the isolated loops of colon. Patients were given a low residue diet and vitamin K parenterally.

In order to get a rough idea of the sensitivity of the intestinal flora upon which to base antibiotic therapy, blood agar plates were inoculated with swab cultures of the feces and tested by a filter paper disk contact method for sensitivity to aureomycin and chloromycetin.\*

\*A discussion of this method will be the subject of a separate report.

each Warburg manometric vessel contained 0.02 ml. of cells and 3.0 ml. of 0.025M sodium bicarbonate containing 0.0342M magnesium chloride. In each instance the substrate was recrystallized acetylcholine bromide in a final concentration of 0.015M. The enzyme was tipped from the side arm after ten minutes of equilibration with 5 per cent carbon dioxide in nitrogen. Manometric readings were taken over a forty-minute period, the temperature being maintained at 38° C. The electrometric procedure was carried out as described here, the temperature of the reaction being 25° C.

The mean value of the cholinesterase activity by the electrometric method was 0.703  $\Delta$  pH per hour for plasma and 0.753  $\Delta$  pII per hour for red cells. The corresponding values by the manometric method were 226.0 microliters per hour for plasma and 253.0 microliters per hour for red cells. Relative activities were calculated for both methods by dividing individual activities by the appropriate mean values. The differences between the relative activities determined by the electrometric method and the corresponding relative activities by the manometric method were calculated. The standard deviation of the differences was 5.49 per cent for plasma cholinesterase and 5.50 per cent for red cell cholinesterase.

*Nonenzymatic Hydrolysis Correction.*—The nonenzymatic hydrolysis correction,  $b$ , was determined by measuring the pII change with time in solutions containing buffer, acetylcholine, and inhibited cholinesterase. Diisopropyl fluorophosphate (DFP) (10  $\mu$ g per milliliter of diluted cells or plasma) was used to inhibit the cholinesterase. The pH was determined at intervals of several hours and  $b$  was then calculated by subtracting the observed pII from 8.00 and dividing by the time in hours.

A slow lowering of pII occurs in the solution of the buffer and enzyme in the absence of substrate and is included in the  $b$  correction.

*Correction Factor  $f$ .*—The values for the correction  $f$ , given in Table I, are based on samples taken from ten subjects. To determine the values, activity measurements were made as described in the Methods section, but pII readings were taken successively on each sample at approximately ten-minute intervals over the pH range from 8.00 to 6.00. The value of  $f$  at pH 7.00 was arbitrarily set equal to 1.00. Using Equation 1, the values of  $f$  were calculated for the selected pH range.

#### DISCUSSION

The advantages of the electrometric pII method are simplicity, a requirement for a minimum of equipment, and the possibility of doing a large number of determinations in a relatively short time. The pII readings may be made easily in a time of one minute for each sample. It is therefore possible to do a large series of determinations in a few hours by adding the substrate to the samples at one-minute intervals.

After this method had been developed, a similar procedure by Croxatto and associates<sup>7</sup> was found in which rate of pII change is used as a measure of serum cholinesterase activity, the pII being measured colorimetrically in a Pulfrich photometer. This method is applicable only to relatively uncolored solutions.

## SUMMARY

1. An electrometric method for plasma and red cell cholinesterase activity is presented in which the enzymatic release of acetic acid from acetylcholine is measured in units of pH change per hour, in a solution of standard buffer capacity.

2. The method has been compared with the standard manometric procedure. The standard deviation of the differences between the methods was 5.49 per cent for plasma cholinesterase and 5.50 per cent for red blood cell cholinesterase.

The author wishes to thank Mr. P. Zvirblis and Mrs. P. D. McNamara for their assistance in this work.

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# STERILIZATION OF DEFUNCTIONALIZED LOOPS OF COLON IN PREPARATION FOR ANASTOMOSIS WITH OTHER VISCERA

## A METHOD OF STUDY AND APPROPRIATE SELECTION OF ANTIBACTERIAL AGENTS

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IN ADMINISTERING intestinal antiseptics for surgery of the large bowel a reduction of the bacterial count of the feces, timed to be at a maximum on the day of operation, is desirable. Two patients were recently treated with a combination of antibiotic and chemotherapeutic agents to prepare the colon for anastomosis with other viscera. At operation full thickness segments of colon approximately 1.0 cm. square were excised from the operative site for aerobic and anaerobic bacteriologic cultures. In the first case the cultures revealed no growth. Forty-eight hour cultures from the second case were sterile, but at the end of seventy-two hours rare colonies of *Monilia albicans* and diphtheroid bacilli were recovered. All organisms in the pretreatment cultures of colonic contents, which showed the usual profuse mixed intestinal flora, were absent (Table I).

The first patient (R. I.) was a 61-year-old man with recurrent obstructing carcinoma of the esophagus following a previous transthoracic resection at which time an extremely short jejunal mesentery was noted. After preliminary gastrostomy for the obstruction, a right transverse colostomy was done to facilitate the preparation of his bowel. On April 13, 1949, Dr. R. H. Smithwick did a transthoracic resection of the recurrent carcinoma and because the short jejunal mesentery precluded an esophagojejunostomy, the transverse colon was used for an esophagocolostomy. At a second stage the continuity of the gastrointestinal tract was re-established, leaving a transplanted segment of colon within the thoracic cavity.

The second patient (W. O.) was a 61-year-old man with carcinoma of the anterior wall of the rectum. During a preliminary cystoscopy the instrument was inadvertently passed through the posterior urethra into the rectum through the carcinomatous mass. Because of this a transverse colostomy was done the same day and it later proved useful in preparing the bowel for surgery. On May 26, 1949, a resection of the rectum, bladder, prostate, seminal vesicles, and posterior urethra with uretero-intestinal anastomosis was done.

Both of these cases will be reported fully at a later date, but the unexpected bacteriologic findings have prompted this preliminary report on the method used for study and preparation of the bowel.

### PLAN OF STUDY AND ANTIBACTERIAL TREATMENT

Therapy was planned to secure reduction in the flora of the colon, timed to be maximal on the day of operation. The choice of antibiotic agents was guided by the sensitivity of the intestinal flora in each instance. Cultures taken just before operation were used to work out the sensitivities to various antibiotics of the organisms remaining in the colon as changed by the intes-

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From the Surgical Service and the Smithwick Foundation, Massachusetts Memorial Hospitals, and the Department of Surgery, Boston University School of Medicine.

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	<i>Bacillus pyocyaneus</i>	+				+				0		
Preoperative day		10	9	8	7	6	5	4	3	2	1	
Coliform count/gram of feces		10,000								0	0	0
Case 2 (Patient W. O.)	<i>Staphylococcus albus</i>	+				+				0		
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	<i>Proteus vulgaris</i>	+				0				0		
	<i>Bacillus mucosus capsulatus</i>	+				0				0		
	<i>Monilia albicans</i>					0				+		
	<i>Diphtheroid bacilli</i>					0				+		
	<i>Bacillus subtilis</i>					+				0		
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Coliform count/gram of feces		10,000	12,000					30,000	5,500	0		

tinal antiseptic therapy. Armed with this information in advance, it should be possible to give systemic or topical treatment with greater speed and specificity for an infection which might develop from the colon after operation. Because of the rapid development of resistance of certain organisms to streptomycin, it was decided to refrain from using it as an intestinal antiseptic but to reserve it for later use in case infection developed.

Cultures of colon contents were obtained through the distal limb of the colostomy. A sterile rubber tube was inserted as far into the bowel as possible and a long slender pipette passed through this tube was used to aspirate colonic juice. All cultures were planted on blood agar plates and inoculated into 6.0 c.c. of beef heart infusion broth enriched with sheeps' blood and fortified with 5.0 mg. per cent of para-aminobenzoic acid, each inoculation consisting of a 2 mm. platinum wire loopful of the juice. Tubes of chopped meat media fortified with infusion broth and sealed with petroleum jelly and paraffin were used for anaerobic cultures. Coliform counts were done by a modification of the streak method described by Poth.<sup>1</sup>

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In order to get a rough idea of the sensitivity of the intestinal flora upon which to base antibiotic therapy, blood agar plates were inoculated with swab cultures of the feces and tested by a filter paper disk contact method for sensitivity to aureomycin and chloromycetin.\*

\*A discussion of this method will be the subject of a separate report.

Twenty-four hour broth cultures diluted  $10^{-6}$  were also tested by a serial dilution technique against these same two antibiotics with the idea of using the most appropriate one as indicated by these sensitivities. The chosen antibiotic was given together with Sulfathaladine as an intestinal antiseptic. It was hoped that this combination would be effective against both gram-negative and gram-positive organisms. The results of these and the subsequent sensitivity tests are indicated in Table II. In the presence of an enterostomy or diarrhea or when enemas or purgatives are to be used, Sulfathaladine is preferable to Sulfasuxidine because of its greater bacteriostatic activity<sup>1</sup> and therefore it was chosen in these cases. For the first patient (R. I.) chloromycetin was selected rather than aureomycin because, although the flora was within range of sensitivity to both antibiotics, aureomycin would have been more likely to cause nausea and the patient was in precarious fluid and electrolyte balance. Inasmuch as chloromycetin was also within the effective range of sensitivity, it was selected to be given with the Sulfathaladine. In the second patient (W. O.) the flora was within the range of sensitivity to both antibiotics so aureomycin was selected because it was more readily available. Administration of the chosen agents was then started via all available routes. Large doses were given as colostomy and rectal irrigations, and also, because of the possibility of "spill over" contamination from the proximal colostomy loop, these drugs were given through the gastrostomy and by mouth as well. Table III indicates the dosage and routes of administration of the various agents.

On the third day before operation, cultures from the colonic juice were obtained to work out the sensitivities of each remaining organism as changed by a period of therapy in order to better cope with infection, should it occur (Table II).

On the second or first preoperative day it was desired to administer prophylactic penicillin and since Poth and co-workers<sup>2</sup> have shown a definite penicillin-Sulfathaladine antagonism affecting certain gram-negative organisms, the Sulfathaladine was discontinued and Sulfasuxidine was substituted. This allowed the unimpeded action of penicillin for the last one or two days before operation after having taken advantage of the superior action of Sulfathaladine during the major part of the preparation. Intramuscular penicillin was then started, and to the first patient (R. I.) penicillin aerosol and lozenges also were given in an attempt to cut down the gram-positive flora in the nasopharynx, throat, and esophagus.

The daily dose of Sulfathaladine and Sulfasuxidine was crushed and suspended in saline in which the antibiotic had been dissolved. This was then instilled into the colostomies, gastrostomy, and rectum in equally divided doses at 6 and 10 A.M. and 2, 6, and 10 P.M., the volume of each instillation being about 100 to 200 cubic centimeters. The last day's dose was dispensed in a very small volume (10 c.c. for each instillation) so that the bowel would be dry at operation. Oral medications were given in equally divided doses on the same schedule.

The following is a summary of the plan of study and treatment in outline form.

1. Reserve streptomycin for later use if infection should develop.
2. After the colostomy is open, culture the transverse colon. Test the mixed cultures for sensitivity against available antibiotics suitable for oral administration.
3. Give colostomy and rectal enemas and magnesium sulfate instillations until the bowel is clean; then use appropriate antibiotic agent along with Sulfathaladine for instillations into the bowel.
4. Three days before operation, culture the mid-colon and work out sensitivities of the constituents of the flora as now altered by therapy to various antibiotics.
5. Two days before operation, omit Sulfathaladine and substitute Sulfasuxidine. Start intramuscular penicillin, penicillin aerosol, and penicillin lozenges.

TABLE II. SENSITIVITY TEST RESULTS

CASE	ANTIBACTERIAL AGENT TESTED	INITIAL MIXED CULTURE	PEROPERATIVE CULTURE—INDIVIDUAL CONSTITUENTS											
			SERIAL DILUTION METHOD						FILTER PAPER DISK METHOD					
			SERIAL DILUTION METHOD	FILTER PAPER DISK METHOD*	B. PYO-CYANEUS	A. AERO-GENES	NONHEMO-LYTIC STREPTOCOCCUS	ALPHA HEMOLYTIC STREPTOCOCCUS	B. PYO-CYANEUS	A. AERO-GENES	NON-HEMOLYTIC STREPTOCOCCUS	ALPHA HEMOLYTIC STREPTOCOCCUS		
1 (R. I.)	Auromycin	γ/ml.	1.5	250-500	10	3	30	0.1	500-1,000	250-500	125-250	6-125		
	Chloromycetin	γ/ml.	6.25	50	25	6.25	6.25	6.25	50	50-100	50-100	50-100		
	Streptomycin	U/ml.			15	7.5	15	7.5	0-20	0-20	250-500	100-500		
	Penicillin	U/ml.			1,000	250	1.5	3.5	----	1,000	20-100	0-20		
	Bactracin	U/ml.			1,000	125	>0.3	>0.3	>1,000	500-1,000	0-20	0-20		
	Sulfamylon	%			0.3	0.6	0.15	0.07	0.06	1.25-2.5	0-0.6	0-0.6		
	Urethane	%			5.0	5.0	5.0	5.0	20	20	20	----		
	Urethane	%			2.5	2.5	0.01	0.007	10	10	0-1.25	0-0.6		
	Penicillin	U/ml.			250	250	1.25	0.7	1,000	1,000	0-125	0-20		
	Streptomycin	U/ml.			1.5	6.0	3.0	1.5	0-25	0-20	25-50	25-50		
2 (W. O.)	Sulfamylon	%			0.03	0.15	0.07	0.003	0-0.6	0-0.6	0-0.6	0.6-1.25		
	Auromycin	γ/ml.	5	125-250	0.7	10	0.1	7	0-10	250-500	0-10	500-1,000		
	Chloromycetin	γ/ml.	3.15	50-100	6.25	3.15	3.15	25	25-50	50-100	0-12.5	25-50		
	Streptomycin	U/ml.			15	63	32	135	20-100	1,000	100-500	20-100		
	Penicillin	U/ml.			0.15	3.5	0.07	1,000	0-2	20-100	0-20	1,000		
	Bactracin	U/ml.			0.3	0.7	12.5	500	0-20	20-100	100-500	1,000		
	Sulfamylon	%			0.07	0.01	0.007	2.5	0-0.6	0-0.6	0-0.6	2.5-5		
	Urethane	%			5	5	2.5	5	25-50	25-50	12.5-25	25-50		
	Urethane	%			0.003	5	0.0015	5	0-1.25	0-1.25	0-1.25	10		
	Penicillin	U/ml.			0.3	500	0.15	500	0-125	0.125	0-125	1,000		
	Streptomycin	U/ml.			3	0.3	0.15	100	0-25	0-25	0-25	50-100		
	Sulfamylon	%			0.07	0.007	0.03	2.5	0-0.6	0-0.6	0-0.6	1.25-2.5		

\*Direct swab inoculation onto blood agar plate. Ten minute contact with disk wet with testing solution. No preliminary incubation. Results in two figures indicate the concentration that bracketed the actual sensitivity which lies somewhere between them.

TABLE III. DAILY DOSAGE AND ROUTES OF ADMINISTRATION OF ANTIBACTERIAL AGENTS

PREOPERATIVE DAY	0	1	2	3	4	5	6	7	8	9	DAY OF OPERATION
By mouth or gastrostomy											
Sulfathaladine (Gm.)	5	5	5	5	5	5	5	5	5	5	0
Sulfasuxidine (R. I.) (Gm.)											0
Chloromycetin (Gm.)											0
Sulfathaladine (Gm.)											0
Sulfasuxidine (W. O.) (Gm.)											0
Aureomycin (Gm.)											0
Crysticillin (units)											300,000
Penicillin (units)											300,000
Penicillin aerosol (units)											0
Penicillin troches (units)											0
Crysticillin (units)											300,000
Penicillin (units)											600,000
Instilled into colonostomy or rectum											
Sulfathaladine (Gm.)											0
Sulfasuxidine (R. I.) (Gm.)											0
Chloromycetin (Gm.)											0
Sulfathaladine (Gm.)											0
Sulfasuxidine (W. O.) (Gm.)											0
Aureomycin (Gm.)											0

\*Case 2 (Patient W. O.) was being treated with aureomycin, Sulfasuxidine, and procaine penicillin (crysticillin) for the urethral perforation before the sixth preoperative day when the regime for preparation of the bowel as outlined in the text was begun.

†Given by error.

‡Oral medication omitted because of marked skin irritation around colonostomy.

## DISCUSSION

When a culture is taken from a bowel being prepared with intestinal antiseptics, some of the antiseptic agent is transferred to the media along with the bacteria and might prevent growth of viable organisms. This is particularly true on solid media and perhaps less so in liquid media. The cultures referred to in Table II are broth cultures and since the media contained para-aminobenzoic acid which inhibits the sulfonamides, it is unlikely that the small amounts of Sulfathaladine or Sulfasuxidine transferred to the broth had any bactericidal action. With the doses of chloromycetin and aureomycin employed, it would seem likely that these antibiotics were diluted beyond their effective bactericidal concentration by the broth. Objections grounded on the transfer of antibacterial agents to the media are largely theoretical because contamination in the operative field would likewise transfer such bactericidal amounts of these agents. It would therefore appear that a practical if not absolute sterilization of the colon has been achieved. It is possible that the appearance of rare colonies of *Monilia albicans* and diphtheroids in the seventy-two hour culture of the second case represents contamination inasmuch as the bacteria did not grow in the original cultures. It is hoped that this type of preparation of the bowel may further reduce the risk of infection and perhaps extend the scope of the surgical usefulness of the colon.

When tested by the serial dilution technique, the mixed flora in the cases reported here were sensitive to concentrations of chloromycetin and aureomycin ranging from 1.5 gamma per cubic centimeter to 6.25 gamma per cubic centimeter. However, preliminary data being collected by Hewitt<sup>3</sup> would seem to indicate that some organisms commonly encountered in the gastrointestinal tract are resistant to much higher levels, especially the *Pseudomonas* group in which many strains are resistant to 50 gamma per cubic centimeter or more. The effective sensitivities for aureomycin are more difficult to determine because this drug is quite unstable and is affected by a variety of substances and media.<sup>4</sup> With a twenty-four hour serial dilution sensitivity technique the values seem to approximate those of chloromycetin, the *Pseudomonas* again being relatively resistant.

The technique described for taking cultures from the colon with a pipette passed through a rubber tube is not satisfactory. The apparatus cannot be manipulated far enough into the colon and contaminants are carried inward from the presenting stoma of the colostomy. It is probable that the antibacterial effect deep within the limb of the colon where there is puddling of the solution is greater than in the segment than can be reached by this technique. Other methods are being studied.

Because no infection developed in these patients it was not necessary to use the information gained from the preoperative cultures and sensitivity studies. It is believed that such advance information would make possible a more rapid and intelligent choice of antibacterial agents. This is especially true in extensive operations involving potential contamination of areas par-

ticularly vulnerable to infection, such as retroperitoneal spaces and the thoracic cavity, from which cultures cannot be obtained postoperatively until infection has been established and drained.

## CONCLUSIONS

A plan of study and antibacterial treatment for preparation of the colon for surgery is described.

In one patient thus prepared, a seventy-two hour broth culture of an excised segment of colon taken from the operative site yielded no growth. In a second patient, rare colonies of *Monilia albicans* and diphtheroids were recovered, but all of the original intestinal organisms were absent.

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## A TECHNIQUE FOR THE COLLECTION OF LYMPH FROM THE RIGHT THORACIC DUCT IN DOGS

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STUDIES of experimental pulmonary edema have been frequently handicapped by the lack of a method for the detection of edema in intact and functioning lungs, particularly when the transudation was occult or of low intensity. In their studies of pulmonary lymph, Drinker and associates<sup>1,2</sup> noted that the flow of lymph from the lungs was increased when congestion was induced. We have recently investigated the outflow of lymph from the lungs in relation to congestion and edema of the pulmonary tissue from experimental heart failure.<sup>3</sup> These observations have indicated that the production of lung lymph is always increased with pulmonary engorgement and edema, and that the quantity of flow is greater with more intense grades of transudation.

It has been shown<sup>1</sup> that the pulmonary and cardiac lymph passes through lymph nodes lying along the superior vena cava. The fluid then courses from these nodes to the right subclavian vein through one or more lymphatics comprising the *right thoracic duct*. In some animals, the pulmonary lymphatics pass to the left, anastomosing with the thoracic duct or entering directly into the left subclavian vein. Drinker and co-workers<sup>1</sup> have collected the pulmonary lymph by cannulation of the right thoracic duct. However, the procedure was so difficult as to be "accomplished satisfactorily in about one out of five animals." In our experience, the cannulation technique has had serious drawbacks. In many animals, the lymphatics were found to be too small to cannulate. In others, cannulation was successful but the lymph failed to flow because of mechanical obstruction by the cannula.

We have employed a simple method for the collection and measurement of right thoracic duct lymph which avoids the pitfalls of minute lymphatics and possible artificial obstruction of the vessels by the manipulation.

### METHOD

The procedure is carried out on dogs under Nembutal anesthesia. To facilitate identification of the lymphatic, 10 c.c. of 1 per cent solution of Evans blue is instilled into the lower part of the trachea through a glass tube. This tube may be introduced through a tracheotomy or through the glottis. A period of ten minutes is allowed for the absorption of the dye into the pulmonary lymphatics. A longitudinal incision is made along

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the right external jugular vein extending to the pectoral muscles and the first rib. The lower end of the jugular vein is then dissected free and followed to its confluence with the axillary vein and the right subclavian vein\* (see Fig. 1). The subclavian vein, in its mid-portion, is in close apposition to the subclavian artery which arches up from beneath. Although there is considerable variation in the right lymphatic vessels, one or more lymphatics have been found in this area constantly, lying either across the ventral surface of the subclavian vein or between the artery and vein, arching upward from the deeper tissue. If dissection is first carried out in this field, location of the lymphatics requires a very short time; a tedious dissection is usually not necessary.

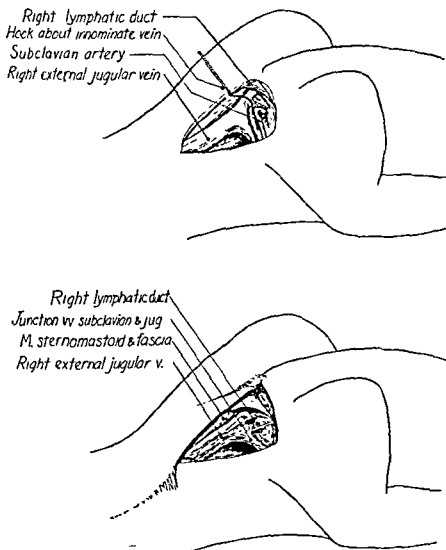


Fig. 1.

Collection of the lung lymph is accomplished by incising the vessel with fine scissors, permitting the fluid to issue freely. The field is carefully wiped dry, and the lymph is then caught on small pledgets of absorbent cotton (10 to 20 mg. in weight) placed over the incised vessel for a measured time. In the properly executed dissection, where all bleeding has been controlled, the lymph is the only fluid absorbed by the cotton. Furthermore, the identity of the lymph is assured by the bluish stain from the dye. We employ a Roller-Smith precision balance to facilitate rapid and accurate weighing of the pledgets.

In normal anesthetized dogs, our method shows that the quantity of lymph from a right thoracic lymphatic vessel varies from 4 to 50 mg. a minute. Drinker and co-workers,<sup>1,2</sup> by cannulation of the lymphatic, find the flow to range from 3.7 to 59.4 mg. a minute. While cannulation is extremely difficult, being unsuccessful in four out of five animals, the pledget technique in all but rare instances is easily accomplished.



## SUMMARY

Anatomic landmarks which facilitate the location of the right thoracic duct are described. By incision of the lymphatic vessel, the fluid is collected on weighed cotton pledgets for controlled periods. The method has proved to be accurate and simple to carry out.

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# PROCEEDINGS OF THE CENTRAL SOCIETY FOR CLINICAL RESEARCH

Twenty-Second Annual Meeting  
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## ABSTRACTS

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### 1. THE DYNAMICS OF COAGULATION

J. GARROTT ALLEN, M.D., PETER V. MOULDER, M.D. (BY INVITATION),  
DANIEL M. ENERSON, M.D. (BY INVITATION), AND  
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Spontaneous (?) bleeding in the presence of hemophilia, prothrombin deficiency, and over-heparinization is not explained by current knowledge or theories of coagulation. Presumably, blood could be extravasated under these conditions because (1) blood is less viscous and its surface tension altered, or (2) the continuity of the walls of the small vessels depends upon some continuing contribution from blood which is destroyed when the clotting process is delayed or inhibited.

Measurements of surface tension and viscosity have shown no significant changes in hemophilia, prothrombin deficiency, or excessive heparinization. Transfusion of normal dogs, until hematocrit readings of 65 to 70 per cent were obtained, resulted in a considerable increase in viscosity and a decrease in surface tension, but these animals bled just as readily from prothrombin deficiency or over-heparinization as did untransfused controls. The irradiated dog (450 r.) given sufficient blood daily to maintain a hematocrit reading of 70 per cent had similar changes in viscosity and surface tension and developed increased clotting time, thrombocytopenia, and hemorrhage. Anemia and hypoproteinemia produced by the daily withdrawal of blood in normal dogs reduced viscosity and increased surface tension but did not cause these animals to bleed or make them unduly susceptible to hemorrhage from Dicumarol or heparin.

Data are presented which suggest that coagulation is a dynamic and protective process. The activity of prothrombin rapidly falls and may disappear entirely fourteen to twenty hours after hepatectomy. Fibrinogen concentrations decline in a similar manner, though less rapidly. That the removal of the liver is not necessary for the rapid decline of prothrombin is indicated by the rapid disappearance of transfused prothrombin in whole blood or fresh plasma. Apparently the turnover of prothrombin is rapid and nearly complete in twenty-four hours, and that of fibrinogen is only slightly longer (two to three days). The fate of these proteins is not known. It is possible that fibrin is constantly formed and is concerned with the maintenance of the integrity of the vascular wall, and that its formation consumes prothrombin.

and winter of 1946-1947 showed . . . against Newcastle virus in two instances (5.5 per cent). . . . sera from thirty-one cases of respiratory infection occurring in the autumn and winter of 1947-1948 revealed antihemagglutinins in ten (32.2 per cent). Many true influenzal infections showed no antihemagglutinins against Newcastle virus. The titer of Newcastle antihemagglutinins was less than 1/100 in all but two instances. All but two of the same sera showed a higher titer of antihemagglutinins against some strain of influenza virus.

Specimens of serum were collected in September, 1948, from 117 healthy medical students, largely Freshmen, who had come to St. Louis from various parts of the country. Of these specimens only one showed the presence of antihemagglutinins against Newcastle virus.

From the excised lung tissue of a case of virus pneumonia which had been followed by chronic atelectasis of the right middle lobe, we apparently isolated a strain of Newcastle virus. The first series of embryonated eggs inoculated with the tissue showed hemagglutinins in the chorioallantoic fluid in the fourth and subsequent transfers. Chickens inoculated with this material developed symptoms similar to Newcastle disease. Virus was recovered from them. Known Newcastle antiserum neutralized hemagglutinins from egg fluid and chick tissue transfers. However, a second series of eggs inoculated with the human lung tissue failed to give evidence of the presence of virus. The patient's serum was not collected for testing until two months after the lobectomy. At this time it showed a titer of 1/40 against the strain of virus isolated from the patient's own lung tissue. It showed a much higher titer against a strain of influenza virus.

The finding of antibodies against Newcastle virus may mean an exposure to the virus or a nonspecific production of antibodies as a result of reactions to influenza and perhaps other viruses. Our findings do not exclude either possibility.

## 6. PRELIMINARY NOTE ON THE EFFECT OF VITAMIN B<sub>12</sub> ON THE PAINFUL ASPECTS OF NUTRITIONAL NEUROPATHY

WILLIAM B. BEAN, M.D., MURRAY FRANKLIN, M.D. (BY INVITATION), AND  
ADOLPH L. SAHS, M.D. (BY INVITATION), IOWA CITY, IOWA

The complexity of the relationship of specific vitamins or food factors to neuropathies which occur in nutritional deficiency states has increased with the discovery of new vitamins, particularly fractions of the B complex. Formerly it was believed that the neuropathy of beriberi was the result of thiamine deprivation. This has been questioned repeatedly without any clear light being thrown on the specific compound or compounds whose relative or absolute deficiency gives rise to neuropathies. Furthermore, it is possible that nonspecific toxins or specific antivitamin or antienzyme factors may be concerned.

Because of this uncertainty and because vitamin B<sub>12</sub> had succeeded in controlling manifestations of combined system disease in pernicious anemia, we tried vitamin B<sub>12</sub> in three patients with painful neurological disturbances which followed nutritional deficiency or chronic alcoholism and in eleven persons with painful neurological disorders not apparently related to nutritional factors. Two of the three patients were observed on a control diet poor in B complex vitamin factors for a week prior to administration of vitamin B<sub>12</sub> intramuscularly in a dose of 15 gamma, after injections of normal salt solution failed to produce any improvement. The third patient, an alcoholic

addict with diabetes, had required narcotics for relief of pain. In these three instances, following the intramuscular administration of 15 gamma of  $B_{12}$ , very dramatic relief of the pain occurred within thirty to sixty minutes. In the diabetic patient no further narcotics were required for pain during the hospital stay of two weeks. In the others, pain gradually reappeared within three to five days and responded again but less dramatically to a second injection of vitamin  $B_{12}$ . They were then taken off the control diet and fed a nutritious diet high in calories, protein, and vitamins and they continued to show improvement. Eleven patients with brachial neuritis (two), multiple sclerosis (two), diabetic neuritis (two), tabes (one), trigeminal neuralgia (one), monocytic leukemia (one), ruptured disc (one), and alcoholic psychosis (one) were given similar injections of sterile saline and vitamin  $B_{12}$ . In no instance was improvement induced, indicating absence of a sedative or analgesic effect for  $B_{12}$ . Since nutritional neuropathies are relatively uncommon in this hospital, it has not been possible to make more extensive observations. This preliminary report is presented that others may try out this compound in nutritional neuropathies.

## 7. EFFECT OF COLD APPLICATION IN PATIENTS WITH ANGINA PECTORIS

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(INTRODUCED BY JOHNSON MCGUIRE, M.D.)

Cold was applied to various body sites in patients with coronary arteriosclerosis and angina of effort. The electrocardiographic changes and general body response were studied.

One refrigerator ice cube, held in contact with the patient's skin, was used in twenty patients with coronary arteriosclerosis and angina of effort. The ice cube was applied to the following sites in different patients: anterior forearm (ulnar surface at the wrist), sides of the nose, chest (region of the right nipple), and the upper abdomen. In order to avoid local chilling of the heart, the precordial region was not used as a site for the test. While applying the ice cube to the nose, the external nares were allowed to remain patent in some patients and occluded in others, in order to determine the effect of chilling the inspired air.

Three patients with angina of effort showed a 1 mm. depression of the S-T segment in one or more of the limb leads following application of cold. One patient showed depression of the S-T segment in Lead I following application of cold to the right chest, although no pain was experienced at this time. Substernal pain followed application of cold to the nose for two minutes. There was prominent depression of the S-T segment in Leads I, II, III at this time. One patient experienced chest pain following application of cold to the nose and wrist for ten minutes, and the pain subsided in approximately three minutes. The electrocardiographic tracing in the limb leads during the bout of pain showed no significant change. One patient developed trigeminal rhythm following application of the ice cube to the nose for five minutes which persisted approximately seven minutes.

Two patients experienced chest pain during the test and two had a delayed reaction of chest pain. One patient, who showed a negative test electrocardiographically following application of cold, had onset of precordial pain approximately five to seven minutes later. The delayed reaction of chest pain was present in another patient, who reported chest pain upon walking from the laboratory. He previously had suffered no episodes of angina for several months prior to the test.

There were no marked changes in blood pressure response noted in any of the patients studied.

This and other preliminary data indicate that a cold stimulus, particularly following a meal, materially increases the positive tests for so-called "ischemia of heart muscle." This may be of clinical value in detecting coronary insufficiency.

It would appear that the cold stimulation of the external skin of the face, especially about the nose, is one of the factors which may contribute toward initiating attacks of angina in patients walking against a cold wind.

## 8. EVALUATION OF THE ZINC SULFATE TURBIDITY AND TOTAL LIPID DETERMINATIONS IN LIVER DISEASE

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(WITH THE TECHNICAL ASSISTANCE OF LILA DOHM, B.S., AND  
ELIZABETH ROBINSON, B.S.)

In order to assess the clinical value of the zinc sulfate turbidity and total lipid determinations as described by Kunkel, a series of 871 consecutive "liver profiles" were studied. These new turbidity tests were contrasted with the routine liver "function" tests simultaneously performed. The 871 determinations were made in approximately 400 patients, more than half of whom had clinical evidence suggestive of liver disease. Needle biopsy of the liver was performed in many instances.

The zinc sulfate turbidity test was contrasted with the thymol turbidity test. 12.0 units was chosen as the upper limit of normal for the zinc sulfate turbidity, and 5.0 units for the thymol turbidity. The cases were divided into the following groups: cirrhosis (nutritional, postnecrotic, and portal), hepatitis (infectious and homologous serum), obstructive jaundice, carcinoma (primary and secondary), and miscellaneous. The miscellaneous group included patients with nonhepatic disease as well as a few with granuloma of the liver and fatty infiltration of the liver.

The zinc sulfate turbidity was elevated while the thymol turbidity was normal in 19.4 per cent of the determinations. The thymol turbidity was elevated and the zinc sulfate turbidity normal in 5.6 per cent of the determinations.

When the determinations were divided according to diagnosis, the following significant data appeared.

(a) In cirrhosis: the zinc sulfate turbidity was elevated and the thymol turbidity was normal in 16.8 per cent of the tests; whereas the zinc sulfate turbidity was normal and the thymol turbidity elevated in 3.7 per cent. (b) In hepatitis: the zinc sulfate was elevated and the thymol turbidity was normal in 8.5 per cent of the tests; whereas the zinc sulfate turbidity was normal and the thymol turbidity elevated in only 0.7 per cent. (c) In obstructive jaundice and carcinoma of the liver: the zinc sulfate turbidity and thymol turbidity tests did not show the significant differences seen in cirrhosis and hepatitis.

It was felt that the zinc sulfate turbidity test was particularly useful in cases of cirrhosis of the liver.

The values obtained for total lipids using the method of Kunkel were contrasted with the values obtained for cholesterol using the method of Bloor. A total of 167 determinations were so compared. It was seen that the values of

the total lipid and cholesterol roughly paralleled one another. The ratio of total lipid to cholesterol was determined and it was found to be  $3.76 \pm 0.7$ . In 17.9 per cent of the determinations this ratio was exceeded. Most of these were instances of hepatitis and/or cirrhosis. The significance of these ratios is to be discussed.

It was felt that these new turbidity tests provide useful information and deserve inclusion in routine liver function studies.

## 9. AN ANTIDIURETIC SUBSTANCE IN THE URINE OF PATIENTS WITH CARDIAC FAILURE

BERNARD A. BERCU, M.D. (By INVITATION), STANLEY N. ROKAW, M.D. (By INVITATION), AND EDWARD MASSIE, M.D., ST. LOUIS, MO.

Recently, much doubt has been cast upon the conventional explanations of the mechanisms involved during cardiac decompensation. Because of the controversial state of this subject at present, it was felt that examination of other factors involved in water balance would be worth while. Therefore, patients with congestive heart failure were studied for the presence of increased amounts of antidiuretic material excreted in the urine.

Patients with definite heart failure were selected for study. None with associated kidney disease or cirrhosis were used. Urines from normal subjects and patients with mental diseases were used as controls. Urines were acidified to a pH of 5.5 to 6.5 with 3 per cent acetic acid, evaporated at room temperature beneath a fan, and then dialyzed in a cellophane sac for six to twenty-four hours. Urines were concentrated so that a fifteen-minute output sample would equal 2 to 4 c.c., and this amount was used as the test dose. Assay was performed on trained normal female dogs in which water diuresis had been produced by giving 35 c.c. per kilogram body weight by stomach tube. Urine samples were collected through an indwelling catheter. A decrease in urine output following intravenous injection of the test material was used as a measure of antidiuretic activity and this was compared with the change in urine output following the injection of known amounts of Pitressin.

Ten patients were studied. Of these, three had hypertensive cardiovascular disease, three had arteriosclerotic heart disease, three had rheumatic heart disease, and two had cor pulmonale. Nine of these were found to have amounts of antidiuretic substance equivalent to 0.5 to 1.0 m $\mu$  of Pitressin in a fifteen-minute urine sample. None of the controls showed any antidiuretic activity in their urine samples. Investigations now in progress indicate that this antidiuretic material is not the same as commercially prepared Pitressin.

## 10. VICARIOUS EXCRETION BY MEANS OF PERGASTRIC INTESTINAL PERFUSION

LIONEL BERNSTEIN, M.D. (By INVITATION), PHILIP B. O'NEILL, M.D. (By INVITATION), ARTHUR BERNSTEIN, M.D. (By INVITATION), AND WILLIAM S. HOFFMAN, M.D., CHICAGO, ILL.

Pergastric intestinal perfusion was carried out in nine preterminal uremic patients as a means of vicarious excretion. The subjects were placed on a Bradford frame with head-end elevated. The perfusing fluid was introduced into the stomach and collected from a rectal tube after transit through the entire intestine. To prevent overdistention of the stomach and vomiting, suction was

applied at the cardia through a second lumen of the gastric tube. Such a scheme avoided many of the disadvantages of other artificial means of vicarious nitrogenous excretion. It required no elaborate equipment, no surgical procedure, no preparation of sterile solutions, and avoided the danger of thrombosis or peritonitis. It could be instituted with a minimum of delay.

A significant fall in the serum nonprotein nitrogen concentration was recognizable after six to eight hours of perfusion. The nonprotein nitrogen level of the intestinal perfusate was directly proportional to the serum nonprotein nitrogen level and inversely to the rate of flow. The mean intestinal nitrogenous excretion was equivalent to a urea clearance of 13.6 c.c. per minute (range, 6.9 to 21.0 c.c.). At the optimal rate of flow of 2 liters per hour, the clearance was of the order of 21.0 c.c. per minute. In contrast, gastric perfusion alone was only about one-sixth as effective.

Blood levels of creatinine, phosphate, and phenols were not appreciably lowered. Alteration of serum sodium, chloride, and bicarbonate levels generally was toward normal.

Serum potassium levels dropped considerably in all instances, though less so when the potassium concentration of the perfusate was made 40 mg. per 100 cubic centimeters. In prolonged perfusions, the serum potassium might fall to the dangerously low level of 12 mg. per 100 cubic centimeters. The high concentration of potassium in the intestinal perfusate implied the nonosmotic secretion of potassium in the intestinal juice. The total negative balance of potassium was as much as five times as great as the total extracellular content of potassium. The lost potassium was therefore largely intracellular. This made the extent of the fall in serum potassium concentration unpredictable. In perfusions of more than six to eight hours, the potassium loss might be dangerous. This is the limiting factor in the use of this method, which otherwise appeared to be of distinct potential value in prolonging life.

## 11. EXPERIENCE WITH HEPARIN-PROTAMINE TITRATION

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CHICAGO, ILL.

Allen and co-workers first noted that in certain hemorrhagic states a heparinemia-like condition exists which will respond to the parenteral administration of protamine or toluidine blue. They devised a heparin-protamine titration technique to detect this state. Using a simplification of this titration, suitable for clinical laboratory analysis, ninety-one tests were performed using forty-eight patients with various hematologic diseases and twenty-three normal subjects. One cubic centimeter lots of blood rendered incoagulable with 90 gamma of heparin are titrated against selected 0.02 c.c. increments of a standard protamine solution until an end point of coagulation at room temperature in one hour is achieved. Normal end points range from 0.12 to 0.18 c.c. of solution, depending on potency of successive batches. Tests were read as -, 0, +, ++, or +++ according to the relationship in 0.02 c.c. increments of the patient to the control value. Of twenty-three tests run on normal subjects, three were -, eleven were 0, and nine were ±. Thus a ± value is of no significance. Six patients with thrombocytopenic purpura, four with acute leucemia, seven with chronic leucemia, two with aplastic anemia, nine with pernicious anemia, four with polycythemia, three with miscellaneous anemias, five with prolonged roentgen therapy, six with obscure bleeding problems, and one each with multiple myeloma, Hodgkin's disease, and myeloid metaplasia of the spleen were tested. Of these, only three of the four acute leucemias and two of the seven

chronic leucemias showed a significant increase. Interestingly, three of the six obscure bleeding problems showed a - reaction, possibly indicating a compensatory increased tolerance for heparin.

## 12. DISTRIBUTION OF BLOOD TYPES IN THE LEUCEMIAS

WILLIAM R. BEST, M.D. (BY INVITATION), LOUIS R. LIMARZI, M.D.,  
AND HENRY G. PONCHIER, M.D., CHICAGO, ILL.

Of five hundred thirty-two patients with leucemia studied, most of whom have been observed over the past twelve years, Landsteiner blood groups were recorded in one hundred thirty-seven and Rh types in thirty. Four of those with known Landsteiner types were Negro; all the others were white. Of those typed, 42 per cent suffered from acute leucemia, 45 per cent from chronic, 5 per cent from monocytic, and 7 per cent from other types of leucemia. The larger group studied consisted of 31 per cent acute, 52 per cent chronic, 6 per cent monocytic, and 11 per cent miscellaneous varieties. Thus blood groups were recorded in a significantly greater percentage of acute conditions than chronic. The distribution of blood types among the one hundred thirty-seven patients was: O, 39 per cent; A, 41 per cent; B, 13 per cent; and AB, 7 per cent. The distribution of blood types as recorded in over four thousand, five hundred recent, routine blood bank tests is: O, 44.1 per cent; A, 38.6 per cent; B, 13.2 per cent; and AB, 4.2 per cent. If the figures from the cases of leucemia are compared by chi-square with those of the larger hospital group, the probability of differences being due solely to random selection is 0.55. Thus, there is no significant difference of distribution between the two groups. Similarly there is no significant variability of blood type distribution in the smaller groups of chronic myelogenous leucemia, chronic lymphatic leucemia, and the pooled cases of acute leucemia.

Of the thirty patients Rh typed, 77 per cent were positive, 23 per cent negative. Comparison by chi-square with the published figures of 85 per cent positive and 15 per cent negative gives a probability of 0.20, revealing no significant difference between the leucemic and the general groups.

## 13. OCCURRENCE OF HEMOPHILIA IN FEMALES

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CHAPEL HILL, N. C.

Hemophilia is considered ordinarily as a disease of the male sex only, inherited as a sex-linked recessive characteristic through the x-chromosome. Of the possible types of crosses in human hemophilia, only two, conductor female  $\times$  normal male ( $Hh \times HY$ ) and normal female  $\times$  hemophilic male ( $HH \times hY$ ), are well established. Rare instances are reported of matings between conductor females and hemophilic males. In this cross,  $Hh \times hY$ , half of the female offspring should be of the genotype  $hh$  and would be expected to be bleeders. However, authentic cases of female hemophilia have not been described. As a result it has been postulated that the genic combination  $hh$  is lethal, or that the bleeding tendency does not become manifest in females.

Recently a strain of dogs with an inherited bleeding disease was discovered. On the basis of the  $Hh \times HY$  cross, a sex-linked type of inheritance was demonstrated. The clotting defect appears to be identical to that in human hemophilia. As a result of a breeding program carried on in our laboratory, bleeder males have been reared to maturity. This report deals



with the mating of bleeder males with conductor females. Six litters from such matings have been studied. Nearly half of the males were hemophiliac, while the remainder were normal. Approximately half of the females were hemophiliac, while the rest showed no evidence of the disease. The distribution into bleeders and nonbleeders in these litters was close to the expected ratio of 1:1 for the cross,  $Hh \times hY$ .

The clotting defect in the affected animals was the same, regardless of sex. All showed consistently prolonged clotting times, normal bleeding times, normal prothrombin values, and a slow disappearance of prothrombin from clotting blood in spite of normal platelet values. The clotting defect was corrected in vitro by normal plasma or small amounts of thromboplastin. Further studies indicated that the bleeder females, just like the bleeder males, lack a plasma factor required for platelet utilization.

No sex difference in the bleeding tendency was observed. Hemarthroses and subcutaneous hematomas have occurred frequently. Some animals have had evidence of internal bleeding. Hemorrhagic episodes have been controlled by plasma transfusions.

The occurrence of hemophilia in female dogs suggests that the genic combination  $hh$  in human beings is not lethal. Absence of human cases may be due to lack of opportunity for their occurrence, or to inadequate investigation of possible cases.

#### 14. OBSERVATIONS ON THE EPIDEMIOLOGY OF INFECTIOUS HEPATITIS

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The occurrence of infectious hepatitis was studied in two widely separated areas in Wisconsin. One region, in the north-central section, consists of a city of 10,000 inhabitants and surrounding rural communities. The other, 250 miles away in the southwestern section, is a prosperous farming region. The geology of the areas is different. The chief method of study consisted of interviews with each individual who had had the disease and with his suspicious contacts. Most of the cases were discovered in this way. An attempt was made to ascertain pertinent facts concerning the development of each case and to document the individual and environmental factors. Similar interviews were obtained with 60 families in which hepatitis had not occurred. Sanitary surveys were made by the State Board of Health, and other data were obtained relative to terrain, climatic variations, and disease rates. For the purpose of this study, jaundice was made a mandatory criterion for diagnosis.

In the northern area, 168 cases of infectious hepatitis occurred, beginning in January, 1947. In the south, 31 cases had appeared, the first in December, 1945. The disease appeared in all months of the year, with a significantly greater number in winter. The greatest number of cases occurred in patients between the ages of 10 and 30, the disease rarely appearing in preschool children or persons over 50. There was a clear tendency to distribution by families. In the 115 families concerned, there were 199 cases (1.7 persons per family). Of the 579 persons in these families, 34 per cent developed the recognizable syndrome. Members of other families in the vicinity were not involved, even though all activities were shared, so far as could be determined. The occurrence of the disease seemed to present a pattern by groups. In the southern region it was confined to an area of approximately four square miles.

Various environmental factors were studied. The drinking water from wells was found to be contaminated in 30 per cent of homes where cases occurred and in only 5 per cent of homes which had been free of the disease. Other considerations tend to minimize the importance of this as the source for transmission. The occurrence of the disease in localized areas by groups seemed to be the most important aspect of these observations.

# 15. RATES OF TURNOVER AND BIOLOGIC DECAY OF CHLORIDE AND CHLORIDE SPACE IN DOGS DETERMINED WITH THE LONG-LIFE ISOTOPE, $\text{Cl}^{36}$

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The long-life radiochloride,  $\text{Cl}^{36}$  ( $T_{1/2} \approx 2 \times 10^6$  years), made possible the study of the early concentration-time course in the blood serum, biologic decay rates, and rates of turnover of chloride in six normal dogs observed continuously for eighteen to thirty-one days under controlled metabolic conditions. These experiments yielded the following information.

(1) The mean concentration-time course of  $\text{Cl}^{36}$  in the serum for the first sixty minutes after intravenous administration can be expressed by the multiple exponential equation

$$\text{CN}_t = 1250e^{-2.079t} + 680e^{-0.308t} + 144e^{-0.0377t} + 674e^{-0.000175t},$$

where

$\text{CN}_t$  = concentration in the serum at any time  $t$ ;  
 $e$  = natural log.

(2) All the seral chloride leaves the serum each minute, and about one-third of the nonseral chloride returns to the serum each minute. Thus in a dog weighing 4.54 kilograms (10 pounds) with a chloride mass of 5.5 Gm., about 2.75 Gm. of chloride pass into and out of the serum each minute.

(3) The mean  $C_{1/2}$  value for the serum was 2.51 days (range, 2.16 to 3.00), the mean  $U_{1/2}$  3.50 days (range, 2.75 to 4.00), and the mean  $E_{1/2}$  3.40 days (range, 2.75 to 3.80).

(4) The mean recovery was 91 per cent of that injected (range, 83 to 97)—88 per cent (range, 81 to 90) in the urine and 2.6 per cent (range, 0.6 to 5.9) in the feces.

(5) The mean chloride space in "serum equivalents" was 35 per cent of body weight (range, 32 to 38) and the mean total body chloride 6.84 Gm. for a dog weighing 4.54 kilograms.

(6) These and other data to be presented indicate that: (a) The chloride space is larger than previously reported. (b) Chloride cannot be employed as a measure of extracellular fluid space. (c) Chloride is present in considerable quantities outside the extracellular fluids. (d) Man would require a daily intake of 43 Gm. NaCl or 15 to 22 lb. of food to have a diet comparable to that of a dog weighing 4.54 kilograms. (e) A diet of 200 mg. sodium for a man weighing 70 kilograms is equivalent to a diet of 13 mg. for a dog weighing 4.54 kilograms. Thus, the usual studies of low sodium metabolism in small animals with "hypertension" must be evaluated cautiously.

The importance of these studies in planning tracer experiments with chloride and bromide and in understanding the metabolism and excretion of chloride and bromide, edematous states, intoxication due to radiochloride and radiobromide in atomic warfare, and other physiologic processes is evident from the data.

16. ORAL ADMINISTRATION OF VITAMIN B<sub>12</sub> IN PERNICIOUS ANEMIA

## II. STUDIES ON THE NATURE AND SOURCE OF INTRINSIC FACTOR

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Vitamin B<sub>12</sub>, when administered parenterally to patients having pernicious anemia in relapse, behaves in the same manner as does the active principle in liver. However, when vitamin B<sub>12</sub> is administered orally it acts as does extrinsic factor, as Berk and co-workers, Hall and associates, and others have shown. Since patients with pernicious anemia lack intrinsic factor, orally administered vitamin B<sub>12</sub> is not utilized, in most cases at least, unless the intrinsic factor is given also, either simultaneously or within a period of six hours (Castle and associates). If the quantity of vitamin B<sub>12</sub> is kept constant, various unknowns can be tested for the presence or absence of intrinsic factor. We have utilized this procedure with seventeen patients having pernicious anemia in severe relapse. The following data have been gathered from these studies.

(1) Intrinsic factor is present in fresh, Berkefeld-filtered, pooled human gastric juice. (2) It is also present in extracts of hog stomach and duodenum. (3) It is destroyed by heating to a temperature of 63° C. for thirty minutes. (4) It is not destroyed in human gastric juice when the latter is stored at a pH of 1.7 at a temperature of 5° C. for three months. (5) Trichloroacetic acid and cold alcohol (95 per cent) precipitates from human gastric juice (prepared for us by Dr. J. L. Bollman) did not contain the intrinsic factor. (6) In vitro mixtures of pooled human gastric juice and vitamin B<sub>12</sub>, after standing at room temperatures for twenty-four hours, were heated to 70° C. for sixty minutes. When these were administered orally to a patient with pernicious anemia in relapse, no hematopoietic response was elicited. (7) The minimal amount of human gastric juice required to produce optimal hematopoietic responses when administered daily by mouth with 5 or 10 µg of vitamin B<sub>12</sub> has not been accurately determined, but in our experience the daily administration of 75 c.c., plus vitamin B<sub>12</sub>, has given optimal results. (8) Variation in the response to large quantities of vitamin B<sub>12</sub> administered alone by mouth has been noted. One patient failed to show a hematopoietic response to a single oral dose of 1,000 µg of vitamin B<sub>12</sub> concentrate. A second patient exhibited a suboptimal response to a single dose of 75 µg of crystalline material. From this, and similar observations by others, it is possible to theorize that lack of intrinsic factor in patients with pernicious anemia is not absolute.

17. CONGESTIVE HEART FAILURE AND HYPONATREMIA: UN-  
TOWARD EFFECTS OF MERCURIAL DIURESIS

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Recent literature concerning the treatment of congestive heart failure has emphasized the value of dietary salt restriction and the frequent use of mercurial diuretics. One of the complications incident to the rapid diuresis produced by this type of therapy is the profound change in the electrolyte balance.

Such an instance was encountered in the case of a 50-year-old man who became critically ill and comatose as the result of treatment of his severe congestive failure with low salt diet and mercurial diuretics. It was not until three

days subsequent to the appearance of coma, when the plasma chloride concentration was found to be low, that the true pathogenesis of his condition was suspected and treatment with sodium chloride solution seemed justified. The patient improved clinically and this improvement paralleled the restoration of electrolyte balance. Since this initial experience eleven other patients presenting a similar therapeutic problem have been treated.

In patients with well-established congestive failure with edema the extracellular fluid compartment is expanded. The removal of the excess fluid in the extracellular space may be accomplished by a combination of low salt intake and mercurial diuretics. During the excretion of this fluid, however, sodium and chloride are removed at a relatively faster rate than water. Consequently, the sodium chloride values fall and the fluid becomes hypotonic. In the presence of subnormal plasma sodium and chloride concentration the diuretic response to organic materials is inhibited. Clinically the situation which at this point presents itself is one of a patient with chronic cardiac failure who in spite of vigorous treatment is getting worse rather than better and does not respond to mercurial diuretics. Dizziness, drowsiness, muscular pains, and apathy appear, and if correction with adequate amounts of sodium chloride is not made in time, confusion, convulsions, and death may result.

#### 18. METABOLIC CHANGES INDUCED BY SUBTOTAL ADRENALECTOMY RESULTING IN CURE OF CUSHING'S SYNDROME; EFFECTS OF LATER ADMINISTRATION OF ACTH

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A metabolic study was carried out upon a 22-year-old woman with typical Cushing's syndrome proved to be due to bilateral adrenocortical hyperplasia. It affords comparative data in the same person under three separate conditions: (1) before subtotal adrenalectomy, (2) after metabolic and physical normalcy had been established by removal of more than 90 per cent of adrenal tissue, and (3) during stimulation of the adrenocortical remnant with large amounts of ACTH (100 mg. per day Armour Standard). The data include observations upon the metabolism of nitrogen, carbohydrate, uric acid, glutathione, cholesterol, and electrolytes as well as daily excretions of 17-ketosteroids and of "11-oxysteroids."

To be emphasized are the following results and conclusions.

(1) Before operation, fasting blood glutathione averaged 26 mg. per cent (highest value, 28 mg. per cent) and carbohydrate tolerance was greatly impaired. Intravenous reduced glutathione (GSH) abruptly converted the diabetic tolerance to normal, but it quickly reverted to the diabetic type. Four months after operation fasting blood glutathione averaged 43 mg. per cent (lowest value, 39 mg. per cent). Carbohydrate tolerance was then normal and has remained so.

These observations are in accord with our previous reports upon the role of GSH in ACTH-induced diabetes in normal human beings. A deficiency of free sulfhydryl (-SH) groups appears to be importantly involved in the pathogenesis of the diabetes observed in Cushing's syndrome.

(2) Excretion of 17-KS and of 11-OS (both elevated threefold preoperatively) became persistently normal after adrenalectomy. ACTH acting upon the adrenal remnant returned these values to their preoperative levels! They became normal again upon cessation of ACTH.

Upon sufficient stimulation and for, at least, a short period of time, a small remnant of adrenal cortex can give rise to the same quantity of steroidal end products as had been produced by two hyperplastic glands during the active stage of Cushing's syndrome. Conversely, the hyperplastic adrenals of active Cushing's syndrome are not receiving maximal functional stimulation from endogenous ACTH.

(3) Despite an ACTH-induced return of excretory corticosteroids to preoperative levels, some of the preoperative metabolic aberrations failed to reappear. This was true also of the preoperative vascular hypertension.

It is likely that some of the clinical and metabolic manifestations of increased cortical activity are a function of time as well as of intensity, that mildly increased cortical activity of sufficient duration eventually will manifest itself. This may be important with respect to therapeutic uses of ACTH.

## 19. A CLINICAL-PATHOLOGICAL SURVEY OF 108 TUBERCULOUS PATIENTS

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Autopsies were performed on 108 patients who had active tuberculosis. The diagnosis of tuberculosis was established by clinical means in 95 (Group A), but the disease was not recognized before death in the other 13 patients (Group B).

*Group A.*—Tuberculosis was the primary cause of death in 73 of the cases in which it was diagnosed before death, but in 18 patients other diseases than tuberculosis were the cause of death. In 13 of these 18 the nontuberculous disease causing death was recognized clinically; in the other 5 it was not suspected. There were four other patients in this group in whom it appeared that the tuberculous process and another disease were of equal importance in the patient's demise. Adequate attempts were made in the treatment of recognized nontuberculous diseases in this group, but in those five patients who had clinically uncovered diseases early treatment might have helped. There were the following conditions unrecognized before death in the five patients in whom the clinical diagnosis of active tuberculosis was made: carcinoma of the pancreas, heart failure due to arteriosclerotic heart disease, pulmonary infarction, and two cases of pulmonary atelectasis.

*Group B.*—Analysis of the 13 patients who had active tuberculosis discovered only at the time of the postmortem showed that two died primarily of the tuberculous process, one from acutely disseminated miliary tuberculosis and one from tuberculous peritonitis. The other eleven patients had clinically recognizable nontuberculous diseases and were properly though unsuccessfully treated. The clinically unrecognized tuberculosis involved the lungs and respiratory passages in all but one patient in whom primary tuberculosis of the kidney was found at autopsy. The other diseases which caused death in this group were generally so acute, severe, or advanced that treatment of the tuberculous lesion would have been of little avail.

Accuracy of clinical diagnosis involving both the tuberculosis and other associated diseases in the group of 108 patients was 121 (86 per cent) in 141 instances. Active tuberculosis was missed clinically in 13 (12 per cent) of the total group studied. Nontuberculous diseases went undiagnosed pre mortem in 6.5 per cent (7).

Tuberculosis services need to seek consultation from other branches of medicine and surgery, as much as do medical and surgical services need the help of pulmonary disease students. This need should encourage hospitals to provide frequent cross consultations. Close proximity of tuberculous hospital and sanatorium units to other medical and surgical units will aid in this endeavor. Therapeutic advances not only in tuberculosis but in other diseases as well will then be readily available to the patients.

## 20. POLYCYTHEMIA VERA WITH HEPATIC VEIN THROMBOSIS: CASE REPORT WITH SERIAL LIVER BIOPSIES AND APPARENT RECOVERY

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A case is presented of a patient who apparently recovered from spontaneous thrombosis of the hepatic vein (Chiari's syndrome). It is believed to be the first case to be reported in which the patient recovered.

*Case Report.*—A 39-year-old shipyard machinist came to the Ochsner Clinic on Aug. 30, 1948, because of weakness, failure to gain weight, and a feeling of fullness in the abdomen of ten months' duration. Three weeks before admission, edema of the legs and ankles and slowly progressive enlargement of the abdomen were noted.

Physical examination and laboratory studies revealed the combined features of polycythemia vera and severe hepatic disease. The patient had a florid complexion with dilatation of superficial vessels and engorgement of the retinal veins. Spider angiomas were noted over the shoulders and chest. The abdomen was filled with fluid. The edge of the liver could be felt about 10 cm. below the right costal margin and there was considerable enlargement of the left lobe. The spleen was ballotable 6 cm. below the left costal margin. Pitting edema was apparent in both ankles and pretibial areas.

There was pronounced elevation of the red blood cell count, hemoglobin, hematocrit, white blood cell count, and platelets. The serum bilirubin value was elevated to a total of 3 mg. per cent; bromsulfalein test showed 46 per cent retention in forty-five minutes; urine urobilinogen excretion was increased, and prothrombin time was 27.8 per cent of normal.

The patient was hospitalized on September 6 for paracentesis and repeat phlebotomies, and 7 mc. of radioactive phosphorus were given intravenously. Initial liver biopsy revealed considerable engorgement of the sinusoids in the central area of the liver with pressure atrophy and beginning fibrous replacement. A small branch of the hepatic vein was seen in one section with a recanalizing thrombus occupying the greater part of its lumen. Despite repeated low prothrombin times, Dicumarol therapy was instituted. Because of recurrent ascites and hemorrhage with paracentesis a peritoneal button was inserted on October 23. With institution of a low sodium diet and repeated administration of mercurial diuretics the patient improved steadily. The liver and spleen showed progressive decrease in size, and on discharge, Dec. 2, 1948, the patient was free of edema and bromsulfalein retention was reduced to 13 per cent in forty-five minutes and total serum bilirubin to 1.16 mg. per cent.

During the next few months the patient required hospitalization once for slight bleeding due to Dicumarol poisoning and once for an intercurrent acute infection. Since that time he has continued to improve, has gained weight,

### 23. AN EFFECTIVE METHOD FOR THE PREVENTION OF RHEUMATIC FEVER AFTER THE DEVELOPMENT OF A STREPTOCOCCAL INFECTION

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From January through June, 1949, at Fort Francis E. Warren, over 1,650 consecutive cases of exudative pharyngitis and tonsillitis were observed. These cases were divided into control and treated groups of approximately the same size according to Air Force serial number. Treatment consisted of the parenteral administration of crystalline procaine penicillin G (suspended in peanut oil containing 2 per cent aluminum monostearate) in doses of 300,000 units on admission and again in seventy-two hours. Later in the study the dosage schedule was changed to 300,000 units on admission, 300,000 units at forty-eight hours, and 600,000 units at ninety-six hours. Studies for the detection of rheumatic fever were performed between the third and fourth week following the initial infection. Throat cultures and blood specimens were obtained from all patients on admission and at the time of re-examination.

That these infections were almost exclusively streptococcal in origin was established by the presence of group A streptococci in approximately 80 per cent of all the original throat cultures and a diagnostic increase of the anti-streptolysin "O" titer in the convalescent blood of about 80 per cent of the control group.

In the entire group, twenty-seven patients developed symptoms suggestive of acute rheumatic fever during a period of from ten to thirty-five days following the initial streptococcal infection. Of these, a definite diagnosis of acute rheumatic fever was made in nineteen patients, seventeen of whom were in the control group and two in the treated group. A diagnosis of possible acute rheumatic fever was made in eight patients, six of whom were in the control group and two in the treated group.

It is concluded that the adequate early treatment with penicillin of streptococcal infections will prevent rheumatic fever in the majority of cases.

### 24. A COMPARISON OF TUBERCULIN AND ARTHUS TYPES OF HYPERSENSITIVITY; IN VIVO OBSERVATION IN THE RABBIT EAR CHAMBER

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(INTRODUCED BY ROBERT G. BLOCH, M.D.)

Using the rabbit ear chamber technique it has been possible to compare in vivo the difference between tuberculin and Arthus types of hypersensitivity. The rabbit ear chamber provides a thin layer of living vascularized tissue 40 to 50  $\mu$  thick which can be inoculated directly. Microscopic observations can be made at frequent intervals under the highest magnification, and the dynamics of tissue change can be watched and recorded with Kodachrome motion pictures.

The tuberculin reaction was studied in five chambers inoculated directly with old tuberculin. One animal was sensitized with bovine tubercle bacilli, strain Ravenel RV, and the others, with BCG. Dilatation of blood vessels occurred during the first thirty minutes after inoculation and persisted for at least seventy-two hours. Generalized sticking of leucocytes to vascular endothelium developed during the first thirty minutes, persisted for forty-eight to seventy-two hours, but decreased in extent after thirty-six hours. Diapedesis of white blood cells resulted in the accumulation of considerable generalized exudate within two to five hours after inoculation. The exudate increased in density during the first thirty-six hours. In three experiments localized hemoconcentration due to seepage of plasma through damaged endothelium was followed by stasis and complete thrombosis of small venules. This occurred in the regions of densest exudate twenty-two to thirty-six hours after inoculation.

The Arthus reaction was studied in six chambers using both bovine albumin fraction V and horse serum as antigens. Immediately following direct inoculation of antigen into the chamber in the sensitized animal, platelet thrombi formed in venules and washed away in the general circulation. Within fifteen to thirty minutes there was considerable admixture of white blood cells with platelets in the thrombi which continued to form. Twenty to sixty minutes after inoculation sticking of large clumps of white blood cells and platelets to vascular endothelium was observed in many vessels. Simultaneously other vessels thrombosed completely and plugs of platelets and white blood cells could be seen separating areas of densely packed red blood cells. Dense exudate developed in the regions where sticking was marked, but little or no exudate accumulated around vessels which thrombosed early. The peak of the reaction occurred after eight to ten hours, although sticking of white blood cells, vascular dilatation, and infrequent formation of new platelet thrombi could be observed after twenty-four hours.

Endothelial damage characterized both types of hypersensitivity. In the tuberculin type, damage was either primary or initiated by local tissue damage. In the Arthus type it seemed to be initiated by an intravascular reaction.

## 25. ELECTROCARDIOGRAPHIC PATTERNS IN PERSONS OVER 80

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Unipolar multiple chest and limb lead electrocardiograms, in addition to the standard leads, were obtained upon one hundred patients over 80 years of age at the Oak Forest Infirmary and the Illinois Masonic Hospital. The abnormalities are tabulated, the largest groups being: first degree heart block, twenty-nine cases; left ventricular hypertrophy, twenty-nine cases; bundle branch block, thirteen cases; myocardial infarction, eleven cases. The establishment of the cardiac position using the unipolar leads explained the axis deviation found in the standard leads: in fifty-three cases of left axis deviation the heart was in the horizontal or semihorizontal position.

Twenty-one electrocardiograms were considered normal using the criteria established by Myers and associates. Of these only fifteen were normal to careful physical examination. Of forty-five patients considered normal to physical examination, thirty cases showed abnormal electrocardiograms. Arteriosclerosis and varying degrees of heart failure accounted in the majority for the physical abnormalities encountered.



Our patients being older than the patients of most other published series, together with the use of multiple chest leads ( $V_{4R}$ ,  $V_{3R}$ ,  $V_{1-6}$ ), as well as the unipolar limb leads, makes comparison with earlier series difficult. The advantages of the additional leads to electrocardiographic interpretation again become apparent.

## 26. CARDIODYNAMIC AND RENAL STUDIES IN CHRONIC PERICARDITIS WITH EFFUSION, WITH PARTICULAR REFERENCE TO THE MECHANISMS OF FLUID ACCUMULATION

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The cardiodynamic and renal changes in chronic pericarditis with effusion were investigated in the dog with particular reference to mechanisms of fluid retention in relation to congestive heart failure.

Pericarditis was induced with irritative cellophane. Post-mortem morphologic studies revealed chronic nonbacterial pericarditis with effusion, passive hyperemia of the liver, kidneys, and lungs, and anasarca.

The following determinations were done serially on unanesthetized dogs: (1) cardiac output (C.O.), (2) renal plasma flow (R.P.F.), (3) glomerular filtration rate (G.F.R.), (4) Na clearance, (5) plasma volume, (6) thiocyanate space, (7) hematocrit, (8) plasma proteins, (9) central and peripheral venous pressure (V.P.), (10) arterial blood pressure, (11) heart rate, (12) intrapericardial pressure, (13) weight.

As pericardial effusion developed, peripheral and central venous pressure rose. This was the initial change recorded. No dog had an increased plasma volume at this time. In some animals thiocyanate space was normal, in others it was moderately increased. Resting C.O., R.P.F., and G.F.R. were all normal at this time. Na clearances suggested slightly decreased Na excretory rate.

With progression of tamponade, V.P. rose further. Plasma volume was increased in some animals, at control level in others. Thiocyanate space was elevated. In this late phase, resting C.O., G.F.R., and R.P.F. were at control levels. Arteriovenous  $O_2$  difference was increased. Ability to increase C.O. in response to excitement and exertion was severely limited. Blood pressure and pulse pressure were moderately depressed.

Prior to exitus, circulatory collapse occurred, with hypotension, markedly elevated V.P. and intrapericardial pressure, decreased right atrial effective filling pressure. Resting C.O., R.P.F., and G.F.R. were depressed. Na clearance was severely impaired, with prolonged retention of infused saline.

It is concluded from this study that in pericarditis with tamponade: (1.) Venous pressure rises as increased intrapericardial pressure jeopardizes right atrial effective filling pressure. Increased venomotor tone appears to be a key factor in the V.P. rise.

(2.) Elevated hydrostatic pressure leads to edema formation. This occurs despite normal resting C.O., G.F.R., R.P.F., and plasma volume.

(3.) It is suggested that with elevated renal venous pressure, increased tubular Na reabsorption brings about Na and  $H_2O$  retention. Plasma volume is thereby maintained despite increased hydrostatic pressure; edema develops without hemoconcentration. Inadequate C.O. during activity may also contribute to Na retention due to an inordinate fall in R.P.F. and G.F.R.

(4.) Preterminally, a critical level of intrapericardial pressure is reached (10 to 15 cm. of  $H_2O$ ) beyond which V.P. cannot rise to maintain right atrial effective filling pressure and C.O. Circulatory collapse ensues. All mechanisms for Na and  $H_2O$  retention are aggravated. Exitus soon supervenes.

## 27. CLINICAL AND EPIDEMIOLOGIC STUDIES OF MUMPS EMPLOYING THE COMPLEMENT FIXATION TEST

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The complement fixation test has been employed for a clinical and epidemiologic study of mumps. Data have been obtained concerning the persistence of antibodies following infection, the occurrence of unrecognized infections, the diagnosis of suspected cases of mumps and of "aseptic meningitis," and a comparison of the "S" or soluble antigen, and the "V" or virus antigen. The results confirm and extend data presented previously from other laboratories.

Tests on one hundred fifteen single sera employing infected allantoic fluid ("V" antigen) detected antibody in the sera of fifty of fifty-one individuals who gave a definite past history of mumps. This positive correlation is notable because the majority of these persons had had mumps ten to thirty years previously. There were seventeen individuals whose sera contained no detectable antibody and it seems highly significant that fourteen of them had no history of mumps, two had an equivocal history, and only one had had mumps. Sera from thirty-two, or 70 per cent, of forty-six individuals with a negative past history of mumps contained antibody, indicating that inapparent or mild infections are frequent.

Diagnostic tests were made with acute phase and convalescent phase sera from thirty-four patients, with definitive results. Twelve sets of sera showed large increases in titer; eleven of these were from cases of mumps, many with complications, and the twelfth was from a patient in whom mumps was strongly suspected. Twenty-two sets showed no significant change in titer; all were from patients in whom mumps was considered in the differential diagnosis and thirteen were from patients with "aseptic meningitis."

Tests employing "S" antigen indicated, as Henle has reported, that antibody to this antigen usually does not persist as long as antibody to the "V" antigen. "S" antigen afforded no decided advantage over "V" antigen for the detection of mumps with paired acute and convalescent phase sera.

It is concluded that the complement fixation test is a valuable tool for clinical and epidemiologic studies of mumps. In addition, the test has been of practical clinical importance in certain instances, such as in patients exposed during early pregnancy and in professional personnel.

## 28. DETERMINATION OF TOTAL BODY SODIUM IN MAN WITH RADIOSODIUM<sup>24</sup>

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Previous methods for determining the sodium content of the human body have been based, for the most part, on calculations from chemical analyses of various organs and an assumed ratio of these organs to the total mass of the

body. Carcass analysis is obviously more accurate, yet reports of such analyses are available only for the fetus and newborn. Since knowledge of the sodium content of the body should be useful in estimating parenteral fluid requirements, an attempt was made to determine it by the isotope dilution principle.

Intravenously administered radiosodium quickly mixes with intravascular sodium and within two to three hours is in equilibrium (except for cerebrospinal fluid) with extravascular sodium. Equilibration with the sodium of brain and bone takes place more slowly but is believed to be complete in about eighteen hours. When equilibrium is complete, serum specific activity should equal total body specific activity, and from knowledge of the former, total body sodium can be calculated as follows:

$$\frac{\text{Na}^{24} \text{ injected} - \text{Na}^{24} \text{ excreted}}{\text{Serum Na}^{24} / \text{serum Na}^{23}} = \text{Total body Na}^{23}$$

Thirty determinations on twenty-seven healthy young men indicate that total body sodium has an average value of 41 meq. per kilogram. In a series of seventeen children the average values are 43 meq. per kilogram for the older children, 49 meq. per kilogram for large infants, and 82 meq. per kilogram for small infants. Comparison of these results with those of actual chemical analyses from the literature will be made.

## 29. COMPARISON OF THE ELECTROPHORETIC PATTERN OF SERUM AND PLASMA IN LIVER DISEASES WITH SPECIAL REFERENCE TO THE GAMMA GLOBULIN FRACTIONS

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Electrophoretic studies on patients with chronic liver disease revealed marked elevations of the fibrinogen peak and lower than expected levels of gamma globulin. To study this phenomenon, we made a comparative study of sixty-five pairs of plasma and serum from persons having various liver diseases, a miscellaneous disease group and a normal group. Electrophoretic studies were supplemented by chemical protein partition (Wolfson and Cohn), flocculation, and other hepatic tests in all except a few miscellaneous cases.

Chemical analysis did not agree with the marked electrophoretic elevations in fibrinogen, nor were fibrinogen peaks completely obliterated upon electrophoretic analysis of the serum. Total serum gamma globulin was higher than the apparent plasma electrophoretic gamma globulin. The serum gamma globulin appeared electrophoretically as two portions of different mobilities, one migrating in the fibrinogen range. This latter portion accounted for the difference between the serum and plasma gamma globulin and, having a similar mobility to fibrinogen, was buried in that peak in plasma determinations. This gamma<sub>2</sub> fraction added to the plasma gamma globulin gave values similar to those of serum and chemically determined gamma globulin. It also accounted for most of the abnormal electrophoretic fibrinogen elevation and when subtracted from the apparent fibrinogen, chemical fibrinogen values were approached. This gamma fraction is apparently identical to the gamma fraction found by Deutch in normal individuals. In our normal group it formed

approximately 2.4 per cent of total serum protein and 16.1 per cent of total gamma globulin. In acute hepatitis it was somewhat higher than normal, averaging 3.9 per cent of total serum protein and 16.7 per cent of total gamma globulin. In obstructive jaundice it was 4.1 per cent and 24.0 per cent, respectively. In cirrhosis this gamma globulin fraction averaged 7.0 per cent of total serum protein and 22.7 per cent of total gamma globulin. Thus, in chronic liver disease in addition to a total gamma globulin increase, there is a disproportionate increase in the gamma<sub>2</sub> fraction. Fifteen cases of rheumatoid and one multiple myeloma showed expected total gamma globulin increases but little change in the gamma<sub>2</sub> fraction. The serum and plasma concentrations of albumin, alpha and beta globulins, were essentially similar. Although plasma electrophoretic tracings appear to be more characteristic for chronic liver disease than do those of serum, their use in protein partition of liver disease is limited because their inability to separate components of the elevated fibrinogen peak gives false impressions of elevated fibrinogen values and lower than expected globulin values.

### 30. INTRA-AORTIC BLOOD PRESSURE DURING SURGICAL RESECTION AND REPAIR OF COARCTATION OF THE AORTA

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(INTRODUCED BY HOWARD B. BURCHELL, M.D.)

A mobile oscillographic camera was used during nine operations for coarctation of the aorta to record continuously the electrocardiogram, heart rate, respiration, and direct blood pressure in the right radial artery. The pressure in the aorta above and below the stricture was recorded by direct puncture simultaneously with these other variables before and after repair of the coarctation.

Compression of the area of the stricture produced no significant change in the pressure in the radial artery or in the aorta, whereas compression of the left subclavian artery in four patients produced an average increase of 18 mm. Hg in the systolic and pulse pressures in the radial artery and an increase of 17 mm. Hg, systolic, and 15 mm. Hg in pulse pressure in the aorta above the stricture. In spite of an increased pressure above the stricture this maneuver in the three cases studied produced an average decrease in the pressure in the aorta below the stricture of 7 mm. Hg, systolic, and 5 mm. Hg, mean.

In eight patients the average time spent opening the anastomosis was 31 seconds, and during this time the mean pressure in the radial artery decreased an average of 11 mm. Hg.

Repair of the coarctation in six patients was associated with an increase of 34 mm. Hg, systolic, and 22 mm. Hg, pulse pressure, in intra-aortic pressure below the stricture.

In seven patients the onset of the pulse wave in the aorta distal to the stricture before repair was an average of 0.02 second later than in the right radial artery at the wrist, whereas after resection the onset of the pulse distal to the site of stricture preceded that of the wrist by an average of 0.05 second. This was not significantly different from the value obtained from the aorta above the site of stricture.

Correlation between (1) the pressures at operation and (2) preoperative and postoperative direct observations of radial and femoral arterial pressures was not sufficiently close to be evident in this study.

The dynamic responses of the strain gage manometer systems used were studied by measurement of their response to square wave and variable frequency sine wave pressure variations. The response to equal amplitude pressure variations was within  $\pm 8$  per cent of the true pressure change up to 30 c.p.s. Higher frequencies were recorded with diminished sensitivity. The deflection time was 0.02 second and the damping coefficient was 0.62. These dynamic characteristics are superior to those of undamped manometers with resonant frequencies of 150 c.p.s.

### 31. THE EFFECT OF CHOLESTEROL-FREE DIET ON SERUM CHOLESTEROL OF NORMAL AND THIOURACIL-TREATED DOGS

EDWARD D. FUTCH III, M.D. (By Invitation), SHIH YUAN TSAI, M.D. (By Invitation), AND RAYMOND GREGORY, M.D., GALVESTON, TEXAS

Experimental studies in dogs indicate that altered thyroid function is necessary for the production of atherosclerosis by cholesterol feeding. In order to elucidate the importance of dietary cholesterol, the following experiment was devised.

Weekly weight and serum cholesterol determinations were made on two groups of four dogs each. After a control period of ten weeks on a commercial dog food, both groups were placed on a diet composed entirely of vegetable products. After a second control period of eight weeks, blood samples were drawn from the hepatic vein and right auricle by means of the catheter technique. Animals in group 2 were then given 1.0 Gm. thiouracil daily. The dose was increased to 1.5 mg. at twenty-five weeks.

In two dogs of group 2, after serum cholesterol had increased to twice the highest control value, hepatic vein and right auricular blood was analyzed for cholesterol.

In group 1, average serum cholesterol was 210 mg. per cent for one dog and 130 mg. per cent for the remaining three animals. This fell to an average of 176 mg. per cent for Dog 1 and 100 mg. per cent for the others. The fall was steady and progressive, lowest levels occurring toward the end of the period.

Group 2 showed striking increase in serum cholesterol after being given thiouracil for ten days. The serum cholesterol reached an average level of 240 mg. per cent over a period of eleven weeks. This approximately doubled average control values.

Comparison of mixed venous blood from the right auricle and hepatic vein showed no significant difference in cholesterol content before and after thiouracil feeding.

It is concluded that (1) the dog can be maintained in good condition on a cholesterol-free diet; (2) all dogs maintained on such a diet show gradual diminution of serum cholesterol; (3) elevation of serum cholesterol produced by thiouracil is not dependent on exogenous cholesterol; (4) excessive production of cholesterol by the liver was not demonstrated in a limited number of animals; (5) the possibility that shift of cholesterol from various organs to the blood serum occurs is being further investigated.

### 32. THE RENAL CAPACITY OF NORMAL, HYPERTENSIVE, AND CARDIAC FAILURE PATIENTS TO EXCRETE SODIUM

RAYMOND GREGORY, M.D., HARRY LEVINE, M.D. (BY INVITATION), DORIS DEPPENBROCK ADAMS, M.D. (BY INVITATION), AND VERNIE STEMBRIDGE, M.D. (BY INVITATION), GALVESTON, TEXAS

The capacity to excrete an intravenous dose of 20 Gm. of sodium chloride per 70 kilograms of weight was studied in normal, hypertensive, and cardiac failure patients. A salt-free diet and distilled water were given one day before and for the two days of the experiment. The sodium content of two-hour urine specimens was determined for the first twelve hours and for three additional twelve-hour periods for a total of forty-eight hours following the injection of sodium chloride. These studies were done on eight normal, nine hypertensive, and eight heart failure patients. The hypertensive patients were studied only if venous pressure, kidney concentration tests, P.S.P. tests, and blood non-protein nitrogen showed no evidence of renal failure.

Marked impairment of the ability of the kidney of each patient with cardiac failure and slightly less impairment of the kidney of each patient with hypertension to excrete sodium was observed in every one of the periods studied.

### 33. PRELIMINARY REPORT OF EXPERIENCES WITH Rh HAPTEN

TIBOR J. GREENWALT, M.D., MILWAUKEE, WIS.

(INTRODUCED BY MAURICE HARDGROVE, M.D.)

Hapten is the term introduced by Landsteiner to designate that portion of the antigen complex which determines its specific reactivity. Separated from the protein portion of the antigenic substance, the haptenic fraction is incapable of calling forth an antibody response when injected into an animal but retains its ability to react with and bind its specific antibody *in vitro*. We have attempted to extract the haptenic portion of the Rh agglutinin from Rh-positive red blood cells with alcohol and ether, using a modification of the procedures described by Carter and Price. This material was assayed *in vitro* by complement fixation, using the method described by Kolmer for the serodiagnosis of syphilis. The hapten material obtained was injected into patients with demonstrable anti-Rh antibodies during pregnancy; 100 to 200 mg of the crude material was injected intramuscularly at weekly intervals.

Sixteen of our patients have terminated their pregnancies. Five received eight or more injections and eleven less than eight injections. Of the five receiving "adequate" therapy, two delivered rh-negative babies, two had macerated, stillborn fetuses at thirty-two to thirty-four weeks, and one delivered an Rh-positive infant with demonstrable antibodies coating its red cells but no clinical evidences of erythroblastosis fetalis. Eleven patients were seen too late and received only 2 to 6 injections of hapten. Two of these delivered macerated, stillborn fetuses, one aborted at eleven weeks, one infant was rh negative, two survived after multiple transfusions, two survived after replacement transfusions, and three showed no clinical or hematologic involvement although they were Rh positive and had positive Coomb's tests.

Definite conclusions cannot be drawn from this material. The anti-Rh titers of our patients receiving hapten have tended to remain low. In spite of this, our results have been discouraging. For example, one patient delivered a macerated fetus during the thirty-fourth week of pregnancy after receiving seventeen injections of hapten. Her titer had never risen above 8 in albumin

and was only 2 three days before delivery. Another patient who had only four haptan injections delivered a normal Rh-positive infant even though antibodies could be demonstrated coating its red cells. One must almost conclude that the haptan therapy was not the determining factor.

#### 34. CARDIOVASCULAR LESIONS IN RATS SUBJECTED TO GROUP A BETA HEMOLYTIC STREPTOCOCCAL PULMONARY INFECTIONS

ROBERT J. GLASER, M.D. (BY INVITATION), GUSTAVE J. DAMMIN, M.D., AND  
W. BARRY WOOD, JR., M.D., ST. LOUIS, MO.

The effect of repeated pulmonary streptococcal infections in the rat was investigated in an attempt to elucidate the nature of the relationship of group A beta hemolytic streptococcal infections to rheumatic fever.

Pneumonia was induced in albino rats by intrabronchial inoculation of group A beta hemolytic streptococci employing techniques previously described. Animals were subjected to from one to seven infections; two strains of streptococci were used alternately in the animals infected repeatedly. Beginning eighteen hours after inoculation, animals were treated with penicillin in amounts previously determined to be adequate for successful control of the infection.

Infections were produced usually at two-week intervals; animals were sacrificed at the end of given periods for study. The hearts were fixed in Zenker-formalin, and microscopic sections were stained with hematoxylin-eosin.

Slight to moderate arteritis and periarteritis of the coronary and/or myocardial arteries occurred in 30 per cent of the experimental animals, whereas 3 per cent of the normal controls exhibited this change. The lesions observed in the experimental group were more intense than those in the control group.

Myocardial lesions, consisting chiefly of pleomorphic cellular foci, were noted in 52 per cent of the animals repeatedly infected; 38 per cent of the controls had similar but less intense lesions.

The incidence of endocardial infiltration by mononuclear cells was slightly more frequent and more intense in the experimental animals than in the controls. Cellular infiltration within the valve substance itself, however, was noted with greater frequency in the control animals. The cardiac lesions observed did not resemble those of rheumatic fever and none of the animals developed arthritis.

Results of this study have demonstrated a significantly greater incidence of cardiac arterial lesions in rats subjected to repeated pulmonary infections with group A beta hemolytic streptococci than in normal control subjects. Single streptococcal infections were not identified with a higher incidence of cardiovascular changes than were found in normal subjects.

The failure of the rat to develop more striking morphologic manifestations of hypersensitivity is in keeping with previous observation. It is believed, however, that the experimental approach used in this investigation, applied to other animal species, may prove a valuable means of studying the problem of the relation of streptococcal infection to rheumatic fever.

#### 35. OBSERVATIONS OF THE CHARACTER OF PLATELETS STUDIED WITH A NEW PHOTOGRAPHIC TECHNIQUE

F. R. HALL, M.D. (BY INVITATION), AND STUYVESANT BUTLER, M.D.,  
CHICAGO, ILL.

In previous reports of Butler, Thomas, and Sanford it was observed that in patients receiving intravenous histamine there was no decrease in the number of platelets, but there did occur a slight but definite decrease in the clotting time

of platelet-free plasma; the higher the histamine concentration, the greater the decrease. It was also observed by Sanford, Butler, and Kennedy that the clotting time of children with hemophilia could be greatly reduced by intravenous histamine.

In order to correlate these observations with the disappearance of platelets and increased coagulation in shock, we surmised that there must be a simultaneous increased production and destruction of platelets at these clinical levels. To study this theory we have given intravenous histamine to patients using techniques described for coagulation studies, but for absolute accuracy, convenience, and a permanent indisputable record, we have made serial photographs every three minutes of platelets in solution for many hours.

Platelets examined in this manner can be studied morphologically with great accuracy and appear to remain constant in number for a long time, and after a period of settling are found to be of a uniform size and characteristic spindle shape.

*Conclusions.*—(1) Platelets are a formed element of the blood. (2) Platelets in solution for many hours in contact with glass do not change their character. (3) A photographic technique for the accurate study of platelets is described.

### 36. THE DIFFERENTIAL DIAGNOSIS OF HYPERGLYCEMIC STATES BY LABORATORY METHODS

G. HAMWI, M.D. (BY INVITATION), AND E. VON HAAM, M.D., COLUMBUS, OHIO

The hyperglycemic state represents a symptom and not a disease and can be produced by various etiological factors. In twenty-three patients in whom a disturbance of carbohydrate metabolism was suspected the glucose tolerance and the insulin glucose tolerance were studied in conjunction with other supplementary laboratory tests. The arterial-venous difference in the glucose tolerance test was taken as evidence of peripheral glucose utilization. Since this utilization is influenced by insulin, a normal arterial-venous difference would imply normal insulin effect. Decreased insulin sensitivity was recognized in the insulin glucose tolerance test. It was taken as evidence of the presence of extrapancreatic factors influencing the carbohydrate metabolism. Using these criteria, seven of the twenty-three cases were recognized as having a normal carbohydrate metabolism. The sixteen patients suffering definitely from hyperglycemia could be divided into the following groups: 4 cases of true pancreatic deficiency hyperglycemia, 3 cases of hyperglycemia showing pancreatic deficiency and extrapancreatic factors, 4 cases of extrapancreatic hyperglycemia, and 5 cases of hyperglycemia with insulin and insulin sensitivity. The latter group may include the nutritional, hepatogenous, and mild functional hyperglycemias.

The hypothesis is presented that patients suffering from carbohydrate metabolism disturbance may change from one type to another, thereby either aggravating or improving the hyperglycemic state. Some confirmation of the foregoing classification was found in the supplementary laboratory evidence and the clinical data available. Patients in whom extrapancreatic influences were postulated as a factor in the hyperglycemia suffered either from obesity or from other demonstrable functional disorders. The possibility that nonglucose reducing substances may interfere with a correct interpretation of the arterial-venous differences is discussed and illustrated with a pertinent case.



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In previous reports of Butler, Thomas, and Sanford it was observed that in patients receiving intravenous histamine there was no decrease in the number of platelets, but there did occur a slight but definite decrease in the clotting time

has been studied over a period of six years. The hemogram, platelet count, bleeding time, clot retraction, tourniquet test, prothrombin level, plasma fibrinogen, serum calcium, plasma vitamin C, and plasma proteins have all been within normal limits on repeated determinations. The administration of large amounts of both fresh whole blood and plasma has not had any effect on the prolonged coagulation time. Precipitins against plasma fractions I and III have not been demonstrable. There was no change in the clotting time of recalcified plasma following centrifugation at high and low speeds. Titration with protamine sulfate revealed no increase in heparin. The presence of a circulating anticoagulant was demonstrated by the prolongation of the clotting time of normal bloods when serial amounts of the patient's blood were added. The anticoagulant effect was also demonstrable in the plasma. Heating at 60° C. for ten minutes inactivated the anticoagulant effect of the plasma. Standing at room temperature or 4° C. for forty-eight hours had little or no effect on the anticoagulant. Thromboplastin assay of the patient's plasma compared with normal plasma and a standard preparation of Maltine thromboplastin revealed low levels of available thromboplastin. Plasma staphylococcus coagulase activator was normal. On two occasions electrophoretic studies revealed the presence of an abnormal fast component of the albumin fraction of the serum. Administration of a course of nitrogen mustard to the patient did not alter the clinical or laboratory findings.

The defect in coagulation in this patient is the result of the presence of an abnormal circulating factor. The findings do not differentiate whether the mechanism is (1) deficiency of thromboplastin, (2) antagonism of a precursor of thromboplastin, or (3) an antienzyme antagonistic to a platelet enzyme necessary for the conversion of thromboplastin precursor to thromboplastin. The end result, however, is a deficiency of thromboplastic activity in the blood which produces hemophilic type of bleeding in the patient.

### 39. CONTROL OF HEART RATE WITH AN INTRACARDIAC THERMODE

HERMAN K. HELLERSTEIN, M.D., AND IRVING M. LIEBOW, M.D., CLEVELAND, OHIO

(INTRODUCED BY HAROLD FEIL, M.D.)

Classical experiments of McWilliam, Flack, and others have shown that the rhythmicity of the S-A node can be altered by thermal changes in excised and perfused hearts and in the exposed heart. We have devised a special thermode which can be placed in the region of the S-A node by venous (jugular, brachial) catheterization of the *intact* experimental animal.

The thermode consists essentially of a 4 x 30 mm. copper U tube attached to the cardiac end of a double lumen catheter. The thermode temperature is regulated by perfusing water of various temperatures (4 to 60° C.) through this closed system. In five Nembutalized, intact dogs, changes in heart rate and in the form of the electrocardiogram were determined by continuous tracings recorded before, during, and after thermal changes. In over 100 experiments, it was possible to vary the rate at will, similar to experiments on exposed hearts.

The most pronounced effects occurred when the thermode was located at the junction of the SVC and right atrium, in the region of the S-A node. There was a latent period of two to twenty-five seconds. Changes in rate were considered to be due to alteration of the temperature of the region of the S-A node. Cold perfusion caused the sinus rate to decrease from the control level of 120 to 140 to 90 to 80 per minute. When the S-A node region was cooled excessively, the pacemaker shifted to the A-V node, with a rate varying from 57 to 86 per

minute. Hot perfusion increased the sinus rate to a maximum of 200 to 232 per minute at 45 to 55° C. The acceleration produced by heating was relatively greater and persisted longer than the slowing produced by cooling.

Marked changes in rate occurred without T-wave alteration. However, in some experiments, when perfusion was rapid, there were T-wave changes which indicated that there had been sufficient temperature change of the blood passing the thermode to alter the rate of repolarization of the subendocardial lamina. Thus, in accordance with our previous observations, cold perfusion produced large negative T waves in cavitory leads and tall positive T waves in extra-cavitory leads; heating produced opposite changes.

The intracardiac thermode may prove valuable experimentally and therapeutically.

#### 40. THE EFFECT OF RUTIN ON THE HEMORRHAGIC PHENOMENA OF EXPERIMENTAL MALIGNANT HYPERTENSION IN THE DOG

H. K. HELLERSTEIN, M.D. (BY INVITATION), AND J. L. ORBISON, M.D. (BY INVITATION), CLEVELAND, OHIO, AND S. RODBARD, M.D. (BY INVITATION), M. WILBURNE, M.D. (BY INVITATION), AND L. N. KATZ, M.D., CHICAGO, ILL.

A striking feature of the malignant phase of experimental Goldblatt hypertension is the occurrence of diffuse hemorrhages. The cause of these hemorrhages is unknown, usually being attributed to degeneration and necrosis of arterioles and capillaries.

In view of the contradictory reports concerning the effects of rutin in reducing capillary fragility and hemorrhage in the malignant phase of hypertension in man, the following study was undertaken. Acute hypertension with uremia was produced by complete bilateral ligation of the renal arteries in sixteen dogs. Rutin was administered subcutaneously, 200 mg. per day, as indicated in Table I.

TABLE I

NUMBER OF DOGS	RUTIN RECEIVED		HEMORRHAGIC LESIONS
	PREOPERATIVELY	POSTOPERATIVELY	
5	0	0	Severe
2	0	Daily	Severe
4	3 days	Daily	Severe
5	10 days	Daily	None

All the animals developed clinical uremia and hypertension, with death in three to six days. The tissues were studied as unknowns.

Severe hemorrhages in the gastrointestinal tract, heart, pancreas, urinary bladder, diaphragm, spleen, and adrenals, together with myocardial inflammation and necrosis, were seen in the first three groups. By contrast, the animals pretreated with rutin for ten days showed complete absence of cardiomyopathy and of the hemorrhagic changes. As a result of the complete obstruction of the main renal arteries, coagulation necrosis of the renal cortex and medulla, with an unexpected sparing of the corticomedullary junction, was noted.

These experiments demonstrate that the hemorrhages usually seen in acute experimental malignant hypertension were prevented by pretreatment with rutin for ten days prior to operation. This protective action may be due to a stabilizing effect of rutin on the ground substance of the arterioles and of the pericapillary sheath. The fact that a shorter period of pretreatment did not provide protection suggests that all our animals were relatively rutin deficient

and that saturation required more than three days of pretreatment. The contradictory reports on the effect of rutin in malignant hypertension in man may also depend upon the relative rutin deficiencies in the patients studied. These experiments call attention to the possibility that the several dietary regimens now being used in the treatment of hypertension may be deficient in rutin and possibly other metabolites.

#### 41. A CLINICAL EVALUATION OF THE BLOOD "SLUDGE" PHENOMENON

JOHN S. HIRSCHBOECK, M.D., AND MAYO WOO, M.D. (BY INVITATION),  
MILWAUKEE, WIS.

The bulbar conjunctival capillaries of a variety of medical patients were observed with the capillary microscope. Erythrocyte sedimentation rates, erythrocyte counts, and serum protein determinations were made within a few days before or after the observation of the circulation in the capillaries. More than 1,200 observations were made.

In general, the degree of "sludging" was directly proportional to the sedimentation rate. "Sludging" was observed in a wide variety of pathologic conditions. Patients with hyperglobulinemia usually had an increased sedimentation rate and well-developed "sludge." Anemia usually caused an increase in "sludging" and sedimentation, whereas erythrocytosis (polycythemia vera or congenital heart disease) always produced an opposite effect.

A discrepancy between the degree of "sludging" and the sedimentation rate occurred in 17.6 per cent of the 619 cases studied. In 5.5 per cent of the cases there was a minimal degree of "sludging" with a rapid sedimentation rate. Pneumonia was the most frequent diagnosis in this group. In 12.1 per cent of the cases well-developed "sludging" with a slow sedimentation rate occurred. Heart failure and hepatic cirrhosis were the most frequent diagnoses in this group. Well-developed "sludging" in the absence of a rapid sedimentation rate is probably the result of stasis in the capillaries.

These observations indicate that blood sedimentation and blood "sludging" have a common cause which, according to Fahraeus and others, is increased rouleaux aggregation.

#### 42. FURTHER EXPERIENCES IN THE MANAGEMENT OF LOWER NEPHRON NEPHROSIS

WILLIAM S. HOFFMAN, M.D., ARTHUR BERNSTEIN, M.D. (BY INVITATION),  
LIONEL BERNSTEIN, M.D. (BY INVITATION), AND PHILIP B. O'NEILL, M.D.  
(BY INVITATION), CHICAGO, ILL.

Lower nephron nephrosis was found to occur after a variety of shocklike episodes, only a few of which were associated with muscle injury or destruction of blood cells. It was seen in lobar pneumonia, acute pancreatitis, acute enteritis, and diabetic coma. The condition was at times difficult to distinguish from other diseases producing oliguria and hematuria, such as acute glomerulonephritis, and bilateral papillary necrosis. Factors producing rapid dehydration and sodium loss, which ordinarily lead to so-called extrarenal azotemia and which respond to fluid and salt administration, could in more fulminating episodes produce true lower nephron nephrosis. The ultimate clinical diagnosis in the patients who survived rested on the finding of severe oliguria from five to thirteen days unresponsive to management, with spontaneous and progressively

increasing diuresis, and with poor renal function even after subsidence of blood nitrogen levels, which function slowly but ultimately returned to normal.

The original illness which brought on the lower nephron nephrosis was important in the prognosis. If infection or trauma persisted throughout the period of oliguria, tissue breakdown was excessive and blood nitrogen levels rose precipitously. These patients died in uremia and heart failure under conditions in which previously well persons might easily survive. All five deaths in our series of eighteen patients occurred with such complications. All patients whose oliguria was associated with self-limiting or controllable conditions, like that of hemorrhage, transfusion reaction, or controllable infection, survived.

We continue to advocate only conservative management in these cases. In the first six cases, successful resistance to the ravages of uremia was achieved by slow induction of edema of nearly normal electrolyte composition. In the later cases, the same results were accomplished by raising sodium levels to normal by intravenous injections of small quantities of 3 per cent sodium chloride and by oral sodium bicarbonate. Thus edema was kept at a minimum. We continue to allow a soft diet ad libitum and believe the slight increase in blood nonprotein nitrogen concentration and edema thereby produced less harmful than the weakness, thirst, and anxiety produced by water and food deprivation advocated by others. Watchful symptomatic treatment of the emergencies of uremia still remains an important factor in successful management.

#### 43. TURBIDIMETRIC DETERMINATION OF SERUM GAMMA GLOBULINS AS CHECKED BY ELECTROPHORETIC ANALYSIS

J. DE LA HUERGA, M.D. (BY INVITATION), AND HANS POPPER, M.D., CHICAGO, ILL.,  
AND MURRAY FRANKLIN, M.D. (BY INVITATION), IOWA CITY, IOWA

Recently a simple method for turbidimetric estimation of the gamma globulins was described by Huerga and Popper. It follows closely the procedure of the thymol turbidity test and is based on recording of a precipitate which forms if serum is diluted with an ammonium sulfate-sodium chloride solution, simulating the chemical determination and partition of gamma globulins by Wolfson, Cohn, and others. The results of the turbidimetric method were found identical with this chemical method. The latter is supposed to give comparable results with electrophoretic partition. Nevertheless, the direct results of the turbidimetric readings for gamma globulins were compared with the results of electrophoretic partition in 163 blood specimens of 134 cases which included normal subjects, patients with various liver diseases, multiple myeloma, and miscellaneous disorders. In 58.1 per cent of the cases the difference between the results of the electrophoretic determination in plasma and the turbidimetric in serum was less than 15 per cent; in 71.5 per cent, less than 20 per cent. The results were not comparable at all in only 3.7 per cent of the cases, varying more than 50 per cent. The mean difference between the turbidimetric and electrophoretic method in per cent was  $17.0 \pm 15.3$ , the electrophoretic method giving slightly higher values on the average. In thirty-seven cases in which both determinations were performed in serum, the mean difference was  $13.5 \pm 8.4$  per cent. The differences were not more marked in pathologic conditions than under normal circumstances. This comparison indicates that the simple gamma globulin turbidity test may replace the electrophoretic determination of gamma globulins in clinical use. The results of the gamma globulin turbidity test are not identical with those of the zinc sulfate turbidity test of Kunkel, especially in hepatic disorders, since the latter may appear depressed in intra- and extrahepatic obstructive jaundice. This depression indicated by an abnormally low ratio between zinc sulfate and gamma globulin turbidity may therefore have diagnostic sig-

nificance. The normal gamma globulin turbidity values range between 0.60 and 1.25 Gm. per 100 ml. of serum. Any elevation above this level is significant. The gamma globulin turbidity values are markedly elevated in liver disorders, especially in cirrhosis and viral hepatitis, and also in rheumatic diseases, several forms of chronic tuberculosis, collagen diseases, chronic skin diseases, and other chronic infections. Comprehension of the exact diagnostic value of elevation of the gamma globulin turbidity in the latter conditions requires additional investigation.

#### 44. AN UNUSUAL CLINICAL PICTURE RESEMBLING PROLONGED SERUM SICKNESS "THOUGHT TO BE CAUSED BY TRICHINOSIS"

JOHN S. HUNT, M.D., NEW ORLEANS, LA.

A young Negro laborer, previously well except for nonseasonal attacks of asthma since childhood, was hospitalized with an illness of one month's duration, characterized by an abrupt onset of fever, migratory arthritis, and severe pain and tenderness in the leg muscles. He had received no serum or drugs prior to this illness. When first seen, the muscle pain had largely subsided, but he had persistent fever and arthritis, and physical examination revealed, in addition, pronounced enlargement of subcutaneous lymph nodes. There was no rash or demonstrable edema. The clinical picture suggestive of serum sickness was supported by laboratory observations of marked leucocytosis; eosinophilia of 4 to 8 per cent; marked elevation of serum gamma globulin, as evidenced by the zine sulfate turbidity test; and the presence of serum heterophile agglutinins, of the Forssman type, in rising titer. Lymph node biopsy in the fifth week of illness showed marked hyperplasia, and muscle biopsy in the sixth week revealed no trichinae or arteritis. There was a strongly positive, immediate reaction to dilute trichina antigen injected intradermally. An electrocardiograph showed changes in T waves and QRS complexes suggestive of myocardial disease. Fever, leucocytosis, and joint pains slowly subsided over a six-week period, and there was a gradual decrease in the size of the lymph nodes. Pyribenzamine treatment during the stage of acute illness resulted in a slight decrease in fever and a definite improvement in the arthritis, pain, and objective joint changes decreasing promptly and recurring explosively, on two occasions, after withdrawal of the drug.

On re-examination, eight months after the onset of the illness, he was asymptomatic and showed, on examination, nothing abnormal except for palpable lymph nodes of much smaller size. The leucocyte count was normal, but with 4 to 5 per cent eosinophiles. Heterophile agglutinins had disappeared from the serum. The electrocardiograph now showed a P-R interval of 0.22 second.

Trichinosis is suggested as the probable cause of this illness on the basis of the muscle pain and tenderness at its onset, the strongly positive intradermal test with trichina antigen, the persistent eosinophilia, and the electrocardiographic abnormalities. This suggests the possibility that, on occasion, the clinical picture of trichinosis may be chiefly one of hypersensitivity to the trichina antigen. In this connection, the report of Reimann, Price, and Herbut on the association of trichinosis with lesions of periarteritis nodosa is of interest.

#### 45. FLUID AND ELECTROLYTE BALANCE IN THE MANAGEMENT OF ACUTE RENAL INSUFFICIENCY

LLOYD T. ISERI, M.D. (BY INVITATION), ALBERT J. BOYLE, PH.D., M.D. (BY INVITATION), THOMAS M. BATCHELOR, M.D. (BY INVITATION), SAMUEL D. JACOBSON, M.D., AND GORDON B. MYERS, M.D., DETROIT, MICH.

Studies of Na, K, Cl, N, and water balance were carried out for periods of four to sixteen days in four patients with severe acute renal insufficiency due to lower nephron nephrosis; studies of sodium and water balance were continued for longer periods in these patients and in six additional ones. Calculations of extracellular-intracellular partition of water, sodium, and potassium were made in the four patients according to the method of Darrow.

Marked fall in plasma sodium was encountered during the oliguric phase as well as during the diuretic phase. The drop during the oliguric phase was due partly to dilution in extracellular fluid and partly to intracellular migration. The drop during the diuretic phase was traceable to the inability of the damaged kidney to retain sodium. Spontaneous resumption of capacity to conserve sodium tended to occur about five to seven days after the onset of diuresis. The importance of following plasma sodium levels and using these as a guide in the treatment will be stressed. Another cardinal feature in the treatment, namely, the daily calculation of fluid balance and utilization of this as an index to fluid intake, will also be brought out.

The plasma potassium did not rise to toxic levels in any of our cases. In one patient who went through ten days of extreme oliguria following circulatory collapse due to diabetic coma, the plasma potassium remained below toxic levels as a result of intracellular uptake of potassium which was released from endogenous protein catabolism. Impairment in capacity to conserve potassium was demonstrated during the diuretic phase and in some cases necessitated administration of supplementary potassium chloride to combat hypopotassemia.

#### 46. THE EFFECT OF X-IRRADIATION ON ANTIBODY FORMATION

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Hektoen, in 1915, first demonstrated that total body exposure of experimental animals to ionizing radiation suppressed the usual antibody response to antigens injected shortly before or shortly after irradiation. Hektoen's observations led him to ascribe this suppression to the destructive effect of radiation on the lymphatic tissue.

This communication relates an attempt to study the capacity of the rabbit to form antibodies during a period in which all lymphatic tissue of the body, with the exception of the spleen or appendix, is essentially destroyed.

Young adult rabbits were given 800 r. total body x-irradiation (250 kv.) twenty-four hours prior to intravenous immunization with sheep cells (1 c.c. of a 2 per cent suspension). Hemolysin titers were determined on serum obtained before immunization and at seven, fourteen, twenty-one and twenty-eight days afterward. Animals thus irradiated and immunized either developed no demonstrable hemolysin titer or developed titers of 1 to 80 or 1 to 40 which were demonstrated on the twenty-first day and the twenty-eighth day, respectively, after immunization. In the normal animal hemolysin titers of 1 to 5120 were produced by the fourteenth day after the intravenous administration of the antigen, diminishing to titers of circa 1 to 160 by the twenty-eighth day. Rabbits given 800 r. total body x-irradiation exclusive of the surgically mobilized, lead-pro-

ected spleen or appendix produce antibodies to this antigen in a manner comparable to normal nonirradiated control animals except that the maximum titer of 1 to 5120 is usually reached on the twenty-first day rather than on the fourteenth. Surgical mobilization of the spleen or appendix was performed while the animals were under Nembutal anesthesia. In the experiments involving spleen protection, the spleen was drawn through a left upper quadrant incision and placed in a lead box, one-fourth inch thick with an opening for the pedicle only, during the period of irradiation. In the experiments involving appendix protection, the method was similar except that the appendix was drawn through a right lower quadrant incision and a cylindrical lead shield, open at one end, was used.

These experiments corroborate Hektoen's original classic findings that antibody formation is suppressed by total body x-irradiation. In addition, it has been demonstrated that if the spleen or appendix of the rabbit is protected by lead shielding during irradiation of the balance of the body, the capacity to produce antibodies to an injected particulate antigen in an essentially normal manner is retained, even though lymphatic tissue elsewhere in the body is largely destroyed.

#### 47. RENAL AND EXTRARENAL DISPOSAL OF CHORIONIC GONADOTROPIN IN THE IMMEDIATE POST-PARTUM PERIOD

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ROBERT B. WILSON, M.D. (BY INVITATION), ROCHESTER, MINN.

The fate of chorionic gonadotropin in the human being has not been well established. Not more than 20 per cent of chorionic gonadotropin administered parenterally to men appeared in the urine.\* Similar experiments in nonpregnant women indicated occasionally much larger urinary recoveries, leading to the view that relatively little destruction or utilization of the hormone occurred in the body.† The foregoing results, however, are not necessarily applicable to the fate of the hormone during pregnancy.

We have studied the renal and extrarenal disposal of chorionic gonadotropin in fifteen pregnant women. Starting immediately on placental delivery, samples of venous blood were taken at three-hour intervals for two or more days. Urine was similarly collected by an indwelling catheter for three or more days. Bioassay of the serum and urine for chorionic gonadotropin was performed by Albert's method‡ The total circulating hormone was estimated at zero time (immediately after the delivery of the placenta) by multiplying the determined concentration of hormone per milliliter of serum by the estimated serum volume. The total amount of hormone appearing in the urine was obtained by addition of the determined values for all samples.

The mean total circulating hormone for the fifteen patients was 36,245 I. U. The mean urinary excretion was 2,105 I. U. Thus a mean of 5.8 per cent of the active circulating hormone was excreted in the urine; 94.2 per cent of it was disposed of extrarenally, presumably by endogenous inactivation or destruction.

This conclusion was further borne out by analysis of the blood and urine curves following placental delivery and by calculations involving the renal clearance of the hormone. In the immediate post-partum period, the renal clearance was 0.47 ml. per minute. At this rate, the serum would be entirely cleared

\*Friedman and Weinstein: *Endocrinology*, 21: 489, 1937.

†Bradbury and Brown: *J. Clin. Endocrinol.* 8: 1037, 1948; Lloyd and co-workers: *J. Clin. Endocrinol.* 9: 268, 1949.

‡J. Clin. Endocrinol. 8: 619, 1948.



of hormone in 106 hours, or cleared to the extinction point of the bio-assay in 89 hours. The blood disappearance curves have a mean half-life of 3.4 hours, or a time for extinction of 9.3 hours, which is grossly incompatible with the time (89 hours) that would be required for reaching the extinction point if the removal of the hormone from the blood depended entirely on renal excretion. Thus, a major route of disposal of the hormone other than renal excretion of the active hormone is present.

#### 48. THE MEASUREMENT OF THE PERIPHERAL CIRCULATION

##### A QUANTITATIVE STUDY

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To date, the clinical measurement of the peripheral circulation is made by indirect means only. The instrumental methods most commonly used are skin temperature studies, oscillometry, plethysmography, photometry, x-ray visualization of the blood vessels, and the use of isotopes.

It is not the purpose of the present paper to evaluate or discuss the relative merits of the various methods but to present a brief report on the results of the measurement of the peripheral circulation by a new method and to discuss the various components of the peripheral records such as amplitude, crest times, diacrotic notch, and the central pulse as related to the peripheral pulse.

Slides will be shown illustrating the quantitative measurement of the pulsatile peripheral circulation in patients with such diseases as hypertensive heart disease, organic occlusive arterial disease, cardiac arrhythmias, and organic valvular disease. A few studies on the effects of smoking in sensitive subjects will be presented.

A special adaptor, for the lantern slide projector, has been made for demonstration purposes. By this means it is possible to project on the screen the circulatory changes from any location in the extremities. During the course of the discussion the circulatory changes from a normal subject will be shown, illustrating the speed and ease with which quantitatively calibrated records can be made.

#### 49. COOMBS TITER VARIATIONS IN ACQUIRED HEMOLYTIC ANEMIA

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The erythrocytes from seven of ten patients with acquired hemolytic anemia have shown agglutinability with antihuman serum rabbit serum (direct Coombs test). Of the three instances of hemolytic anemia with negative Coombs tests, one was a severe but transient episode complicating pneumonia and empyema, one had persisted intermittently for six years in an individual with no family history of jaundice and a normal erythrocyte osmotic fragility, and one occurred as the terminal episode in a patient with Hodgkin's disease.

Two patients with Hodgkin's disease, one with carcinomatosis and one with pernicious anemia, experienced hemolytic episodes associated with a positive Coombs test. Agglutination of their erythrocytes occurred only in low dilutions of rabbit serum and decreased or disappeared with the termination of the episode. Because commercially available antiglobulin serum failed to give similar reactions, the specificity of the local serum was checked against erythrocytes from sixty patients obtained as random samples from the blood bank. Only one

sample, erythrocytes from a female patient hospitalized for transfusion for anemia, gave a similar low titer reaction. Erythrocytes from fifteen anemic patients with carcinomatosis, Hodgkin's disease, multiple myeloma, leucemia, Gaucher's disease, hypersplenism, and pernicious anemia, all without evidence of hemolysis, gave negative Coombs tests.

In contrast to the low titer agglutination observed in patients with symptomatic hemolytic anemia, erythrocytes from three patients with acute idiopathic hemolytic jaundice were agglutinated by high dilutions of antiglobulin serum. The direct Coombs titers persisted at high levels in two patients subjected to splenectomy. The patient without demonstrable serum antibody experienced partial remission; the patient with serum auto- and iso-agglutinins was not benefited. A third patient, without splenomegaly, received nitrogen mustard. This did not alter the Coombs titer or the degree of hemolysis and produced a fatal thrombocytopenia.

It is concluded that not all patients with acquired hemolytic anemia have positive Coombs tests, and that potent antiglobulin serum is necessary to obtain positive tests in certain other cases. In those cases with low titer agglutination, the finding of a positive Coombs test was correlated with the presence of a hemolytic episode. In two patients showing high titer agglutination, splenectomy did not lower the agglutination titer of their erythrocytes in antiglobulin serum but did lead to a beneficial decrease in the rate of erythrocyte destruction in one case. Additional study is necessary to determine the effect of detectable serum agglutinins on the rate of hemolysis following splenectomy.

## 50. CLINICAL EXPERIENCE WITH A NEW ANALGESIC AGENT

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(INTRODUCED BY F. W. MADISON, M.D.)

Clinical trial of a new analgesic, 3-hydroxy-N-methyl-morphinan-hydrobromide (NU-2206), has been carried out. Of thirty-five patients studied, eleven had advanced malignancy, nine were postoperative, and the remaining fifteen had pain from a variety of causes including renal colic, lupus erythematosus, peripheral vascular disease, rheumatoid arthritis, fracture, myocardial infarction, and congestive heart failure. Experience thus far indicates that the drug has approximately three times the analgesic potency of morphine. In seven patients relief of pain was inferior to that obtained when comparable dosage of other analgesics was used. Nausea and vertigo were encountered though less commonly than with morphine, Dilaudid, or Pantopon. When used over a long period of time, tolerance developed and the dosage had to be increased. Euphoria was noted in only four patients. One patient with advanced peripheral vascular disease received fourteen doses of 3 mg. and twenty-six doses of 5 mg. over a period of sixteen days. Following amputation on the sixteenth day, pain ceased and the drug was stopped without withdrawal symptoms. The most striking results were obtained in the group with advanced carcinoma where in many instances pain could be completely abolished without appreciable sedation or respiratory depression even when as large a dose as 20 mg. was given.

No major toxic effects have thus far been observed.

## 51. THE EFFECTS OF COLD ON MAN: SPECULATIONS ON DISEASES OF COLD TEMPERATE CLIMATES, NUTRITION, AND THE PITUITARY-ADRENAL AXIS

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Investigations on healthy young men exposed to the cold have uncovered adaptative changes which might provide clues for understanding the genesis of some diseases most prevalent in cold temperate climates. For example, it has been established for troops in the field that the voluntary caloric consumption is greater the colder the climate. Among men traveling in extreme cold there are, on occasion, alterations in water metabolism and electrolyte balance, creatinuria, retention of ascorbic acid, and other changes reminiscent of those observed in injury and other stress. Recently, metabolic changes were observed in thirty-two heat-acclimatized men abruptly exposed to a cold climate (mean temperature,  $-26^{\circ}$  F.) for twelve days. The results were similar in many respects to those in man after injection of adrenocorticotrophic hormone and to the "general adaptation syndrome" of animals during stress. Statistically significant responses during the first day included hypothermia, diuresis, hemoconcentration, eosinophilopenia and lymphopenia, neutrophil leucocytosis with increase in the percentage of young cells, hyperuricemia, hyperphosphatemia, and hyperkalemia, hypotension and hypochloremia, transient retention of sodium and chloride, and depletion of ascorbic acid in the blood. Most of these changes reverted to normal after two days of exposure. However, in the last six days of exposure there were an increase in kidney and adrenocortical activity, a test dose of water being excreted rapidly at very low specific gravity; continued eosinophilopenia and lymphopenia; and continued hyperuricemia and hyperphosphatemia.

Mills and others have shown that diabetes, hyperthyroidism, pernicious anemia, Addison's disease, and leucemia are most prevalent in cold temperate climates, especially so in populations exposed to wide seasonal and diurnal variations in temperature. There are other chronic disorders such as multiple sclerosis, the rheumatic diseases, and certain cardiovascular diseases which have a geographic distribution similar to that of the five diseases just mentioned. In man, acute exposure to cold has been said to precipitate pneumonia, nephritis, and attacks of gout.

We accept the concept that climatic or environmental stresses are related to the genesis of certain disease processes in man. We would alter the hypothesis of Mills and others that the development of many diseases is directly related to the metabolic load imposed by stimulating climates to the hypothesis that exposure to cold stimulates the pituitary-adrenal axis. There may then occur pathologic changes, some of which have already been detected in studies of the "general adaptation syndrome."

## 52. PATHOLOGIC AND ELECTROCARDIOGRAPHIC STUDY OF THE AURICLES

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(INTRODUCED BY JOHNSON MCGUIRE, M.D.)

By current standards, auricular disease is said to exist when the P wave in any of the standard leads exceeds .12 second in duration, or 3 mm. in height. The validity of these criteria were studied as follows:

1. Two hundred electrocardiograms on normal subjects ranging from birth to 20 years of age were examined. None of the 200 electrocardiograms exceeded the accepted criteria for auricular damage.

2. One hundred electrocardiograms in which the P-wave measurements exceeded the limits mentioned were chosen by random selection. They were classified according to the clinical diagnoses. Abnormal P waves were found in 5 cases of myocardial infarction, 28 cases of hypertensive arteriosclerotic heart disease, 19 cases of rheumatic heart disease, 4 cases of congenital heart disease, 9 cases of cor pulmonale, and in 35 cases comprising a miscellaneous group (beriberi heart disease, thyrotoxic heart disease, etc.). Broad P waves were recorded three times as frequently as tall P waves.

3. Necropsy findings in 122 unselected cases of heart disease were correlated with the P wave in the electrocardiogram. There were 42 cases of myocardial infarction, 28 cases of hypertensive arteriosclerotic heart disease, 25 cases of congenital heart disease, 15 cases of rheumatic heart disease, and 12 cases of cor pulmonale. Pathologic abnormalities of the auricles were found in 58 cases and consisted of dilatation, hypertrophy, intra-auricular thromboses, endocardial thickening, or combinations thereof. P-wave abnormalities were present in 28, or 49 per cent, of these cases. Where electrocardiographic and pathologic findings co-existed, there was no correlation between the type of P-wave abnormality and the particular pathologic change recorded. Electrocardiographic evidence of auricular abnormalities was present in 42 cases of the entire autopsied series. In 14 instances of these 42 cases, or 33 per cent, pathologic confirmation was absent. There were 50 cases in the series of 122 in which neither pathologic nor electrocardiographic evidence of auricular abnormalities was demonstrable.

It was concluded that (1) abnormal P-wave changes may occur in the absence of gross pathologic changes in the auricles; (2) normal P waves may exist in the presence of gross pathologic changes in the auricles; (3) no correlation existed between the auricular chamber involved and the particular lead in which P-wave abnormalities were found.

## 53. THE PRESENCE OF A PHOSPHATASE IN THE HUMAN AORTIC WALL

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The failure to demonstrate a phosphatase in the human arterial wall has been a subject of significance in the discussion of the pathogenesis of arterial calcification. Since previous investigations appear to have been performed only at alkaline reactions, a study was undertaken covering a wider pH range.

After removal of the adventitia and the external part of the media from human aortas obtained at autopsy, the remaining part of the tissue was ground

finely with water in a Pyrex grinder and the samples were centrifuged. For study of the enzymatic activity portions of the supernatant solution were added to citric acid sodium hydroxide and Veronal sodium carbonate buffers, covering the pH range 3.1 to 10.1. Disodium phenyl phosphate was used as a substrate. Duplicate samples, controls, and blanks were run at each pH level examined. The reaction was stopped by the addition of trichloroacetic acid and the phenol content of the filtrates determined by Folin and Ciocalteu's method. The enzymatic activity was estimated by subtracting the color of the controls from that of the samples.

The studies showed a significant phosphatase activity with a maximum at pH 5.7 to 5.8. At this optimal pH level a fair proportionality was observed between the amount of supernatant fluid used and the phosphatase activity and between the reaction time and the enzyme activity.

#### 54. THE DIAGNOSTIC VALUE OF HIGH PRECORDIAL LEADS

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High precordial leads taken at the intersections of vertical lines through the  $V_3$ ,  $V_4$ ,  $V_5$ , and  $V_6$  positions with a horizontal line at the junction of the third interspace and sternum have been obtained on approximately 4,000 patients. The findings in these leads were correlated with those in the customary precordial and Goldberger limb leads and with the pathologic findings in 300 cases that came to autopsy.

Cardiac rotation appeared to have a greater influence on the QRS pattern in high precordial leads than in those taken in the usual positions. Clockwise rotation displaced the transitional zone farther to the left in high than in the customary precordial leads and often led to the registration of an RS pattern, typical of the potential variations of the epicardial surface of the right ventricle, in leads high in the axilla as well as in a  $V_L$ . Counterclockwise rotation tended to shift the transitional zone farther to the right in the high than in the routine precordial leads.

High precordial leads were of particular value in the detection of infarcts localized to the basal portion of the anterior or lateral walls of the left ventricle, as demonstrated by pathologically proved cases with diagnostic signs in high precordial but not in the routine precordial or limb leads.

High precordial leads were also of value in the estimation of the basilar extent of anterior or lateral apical infarcts, as illustrated by cases of localized anterolateral apical infarction, showing diagnostic patterns in the customary but not in the high precordial leads and extensive anterolateral infarction with diagnostic patterns at both levels. The differentiation from high precordial lead patterns due to right and left ventricular hypertrophy and right and left bundle branch block is illustrated.

#### 55. STUDIES ON THE RENAL TUBULAR TRANSPORT MECHANISM FOR GLUCOSE

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GORDON JOHNSON, B.S., AND ELAINE GIANAS, R.Ph., CHICAGO, ILL.

(INTRODUCED BY ROBERT W. KEETON, M.D.)

Recent observations by Conn and co-workers indicate that the administration of adrenocorticotrophic hormone (ACTH) to normal human subjects results in a diabetic state. In addition to hyperglycemia, ACTH is be-

lieved to decrease renal tubular resorption of glucose. Glutathione antagonizes this effect of ACTH presumably by reversing the inhibitory action of ACTH on tubular transport mechanisms.

Hyperglycemia has been reported to effect adrenal-cortical discharge as reflected by a fall in the lymphocyte count in the rat and also in man. Thus hyperglycemia in itself can be considered as a stress phenomenon.

To obtain quantitative data on the effect of adrenal-cortical discharge on the tubular mechanism for glucose, it was planned to do  $Tm_g$  determinations in trained dogs before and after the administration of epinephrine. A preliminary experiment resulted in a 70 per cent drop in the  $Tm_g$  and a 77 per cent drop in the eosinophil count, following the intravenous infusion of epinephrine (5 gamma per kilogram per minute for one hour). Eight periods, fifteen to twenty minutes in duration, were conducted in each experiment over a five-hour period of glucose infusion. The  $Tm_g$  values for the last two to three periods were within normal limits as reported for the dog (200 to 300 mg. per minute). In five of six dogs, the initial two to three periods showed depressions of the  $Tm_g$  ranging from 33 to 59 per cent.  $Tm_g$  values as low as 92 mg. per minute have been obtained under these conditions despite glucose loads of 1.5 to 2.0 times the animal's normal  $Tm_g$  level. In contrast to the gradual rise in  $Tm_g$  as a given experiment progressed, the eosinophil count fell continuously to a low value at the completion of each run.

The marked depressions of tubular resorption of glucose seemed to coincide with high environmental temperatures. This is of interest since it is known that heat stress will fire the pituitary-adrenal mechanism. It is postulated that environmental heat stress in combination with the stress of hyperglycemia may have been responsible for the anomalous  $Tm_g$  observations noted in this series of dogs.

#### 56. AN INDIRECT, QUANTITATIVE METHOD FOR THE ESTIMATION OF HEPARIN ACTIVITY IN VITRO: CLINICAL APPLICATION

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Evidence has been presented which suggests that some hemorrhagic states are due to the presence of excessive amounts of heparin in the blood. In such conditions the antiheparin agents, protamine sulfate and toluidine blue, may be of great value clinically. A laboratory procedure which will permit the selection of patients for this type of therapy and which can be used to control such therapy is desirable. A method is described which utilizes a modified "one-stage prothrombin time" type test. With this technique it is possible to detect the presence of heparin in amounts as small as 3.5  $\mu$ g per milliliter of plasma. Under the conditions of this test the ratio of the concentration of protamine sulfate required to neutralize heparin added in vitro or in vivo is 1.5:1. This ratio appears to be a constant. When the amount of protamine sulfate required to neutralize a certain amount of added heparin is greater than that predicted by this ratio, it is concluded that the excess protamine neutralizes endogenous heparin. The utility of the test in the protamine sulfate therapy of certain hemorrhagic states is illustrated by case histories. In practice it has been possible to predict which patients will be benefited by the use of antiheparin agents. It has also been possible to determine the adequacy of the treatment, even in the absence of frank bleeding.

## 57. THE EFFECT OF ATROPINE SULFATE AND DIBUTOLINE ON THE NOCTURNAL GASTRIC SECRETION IN MAN

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The effect of atropine and of atropine and adrenaline in oil on the fasting gastric secretion was studied in forty-five patients hospitalized for peptic ulcer. Atropine administered parenterally in dosages of 1.0 to 2.0 mg. every 3 to 4 hours exerted a variable effect. A depression in acid secretion occurred in approximately 33 per cent of the studies; the effect was of short duration. Increases in gastric secretion were observed in approximately 26 per cent of the studies. Similar results were obtained with 2.0 mg. doses. The action of atropine upon the parietal cell appeared to be related to the state of activity of the cells at the time of administration of the drug. Of the patients with duodenal ulcer in whom a reduction was obtained, the secretory rate in 66 per cent was less than the average hourly rate observed in such patients. However, a depression in gastric secretion was not obtained uniformly in all individuals with low rates of secretion. The reduction in output of acid in individuals with high secretory rates was associated with severe symptoms of atropine toxicity. Atropine also was administered in doses of 1.0 mg. simultaneously with 2.0 mg. of adrenaline in oil. No additive effect was noted insofar as the volume of gastric secretion was concerned. However, the concentration and output of acid were reduced more frequently by the combination of drugs than with atropine alone.

The effect of a pharmacologically related compound, Dibutoline (Dibutylurethane of dimethyl-ethyl- $\beta$ -hydroxyethyl ammonium sulfate—Merck) on the nocturnal gastric secretion was studied in fifteen individuals. Dosages of 10 mg. given intramuscularly every four hours during the night yielded results similar to those obtained with atropine. Although a decrease in the output of acid was observed more frequently with Dibutoline, the depressant effect, whenever it occurred, likewise was variable and of short duration.

## 58. STUDIES OF BLOOD COAGULABILITY AS INFLUENCED BY DIGITOXIN

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Digitoxin was administered to all subjects used in this study by either the oral or parenteral route. In all instances studies of the coagulation mechanism were performed both before and after digitalization. All subjects used presented no evidence of cardiac or thromboembolic disease.

The bleeding time, prothrombin time, and coagulation time (Lee and White) were determined in five patients. Two of these patients received 0.6 mg. digitoxin intravenously and three received 1.2 mg. digitoxin intravenously. The aforementioned studies were then determined at intervals of five to ten minutes, one hour, four hours, seven hours, and twenty-four hours. In no instance was there any significant decrease in the bleeding time, coagulation time, or prothrombin time.

Eleven patients were then studied with heparin tolerance estimations before and after receiving a full digitalizing dose (1.6 mg.) of digitoxin administered orally over a twenty-four hour period. No significant change in the

heparin tolerance curves was apparent after digitalization when the test was performed according to the method described by de Takats and associates. In order to make the method more sensitive, it was modified by using the Lee and White coagulation time. With this modification, six determinations on five patients revealed an increase in heparin tolerance, and seven determinations on six patients revealed a decrease in heparin tolerance. It is suggested that the wide variations in these data are due to variations intrinsic in the method rather than to the effect of digitoxin.

The problem was then approached by studying the prothrombin times by a modified Quick one-stage technique in twelve subjects. Following digitalization with digitoxin, there were no significant alterations in the prothrombin times in any instance. After digitalization, the subjects were Dicumarolized in the usual fashion. No inhibition of the effect of Dicumarol by digitoxin as measured by the effect on the prothrombin activity was evident.

Three patients were given Dicumarol so that prothrombin activity was reduced to a range of 10 to 30 per cent. After stabilization of prothrombin activity by the administration of maintenance doses of Dicumarol, each of the three patients was digitalized with 1.6 mg. of digitoxin given orally. In none of the three cases did the digitoxin appear to interfere with the effect of Dicumarol as reflected by the prothrombin activity.

Finally, the problem was approached by the determination of the coagulation time both by the conventional Lee and White technique and in tubes treated with silicone for the purpose of simulating the endothelial lining of the vascular network. Ten patients were studied by this method for a three-day control period. It was noted that there was considerable variation in the coagulation times in the same individual from day to day. This variation was noted in both techniques but was especially evident in the technique employing test tubes treated with silicone. These patients were then digitalized by the oral administration of 1.6 mg. of digitoxin, and they were placed on daily maintenance doses of this drug. The coagulation time was studied daily for three days according to the previously mentioned techniques. Again there were no consistent changes in the coagulation time as measured by either technique following the administration of digitoxin.

It is concluded, therefore, that the methods used in this study have demonstrated that digitoxin, administered either orally or intravenously, has no effect on the coagulation mechanism of the blood. Therefore, if digitalization is indicated, this study suggests that there should be no hesitation in carrying out this therapeutic procedure with a view that thromboembolism may result therefrom.

## 59. THE USE OF VIODENUM IN THE TREATMENT OF ULCERATIVE COLITIS

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(INTRODUCED BY ARMAND J. QUICK, M.D.)

A continuous search is being made to find an adequate means of combating the etiological factors of chronic idiopathic ulcerative colitis. Among the more recent additions to our medical armamentarium is Viodenum. This substance is whole desiccated and defatted duodenum, which is believed to contain certain antisecretory and antiproteolytic factors. Thirteen patients with chronic ulcerative colitis in acute exacerbation were treated with Viodenum in addition to the commonly used therapeutic measures. Tablets were administered



only after powder had been given in the initial phase of the treatment and until the acute flareup had subsided. The results are as follows: (1) one patient showed no improvement; (2) exacerbation of the disease was noted in three instances, in one of which the tablets passed through the bowel without disintegration; (3) two patients were forced to discontinue treatment because of intolerance as manifested by nausea, diarrhea, and cramps; (4) definite clinical improvement was observed in six patients; (5) no definite opinion could be reached in two.

## 60. THE EFFECT OF TETRAETHYLAMMONIUM BROMIDE ON THE CARDIAC OUTPUT OF NORMOTENSIVE AND HYPERTENSIVE PATIENTS

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In a previous study we have shown that the fall in arterial blood pressure of normal and hypertensive patients induced by spinal anesthesia is not associated usually with a significant fall in cardiac index. This evidence has been used to support our belief that the cause, or at least the sustaining cause, in essential hypertension is an increased vasomotor tone. In attempting further to elucidate the pathogenesis of essential hypertension, tetraethylammonium was used to lower the blood pressure in patients with essential hypertension, and cardiac output studies were made by the direct Fick method.

Sixty-one cardiac output measurements were made on nine normotensive and eleven hypertensive patients before, during, and after the intravenous injection of tetraethylammonium bromide. The twenty patients were divided into four groups: sedated and unsedated hypertensives, sedated and unsedated normotensives.

In both the sedated and unsedated normotensives tetraethylammonium produced a fall in blood pressure but no significant fall in cardiac index. After recovery from the tetraethylammonium, as shown by the disappearance of tachycardia and the return of the blood pressure to or toward control levels, the cardiac index remained lower than either the control level or the level obtained during the tetraethylammonium effect.

In the unsedated hypertensive group the control cardiac indexes were slightly lower than in the normotensive or sedated hypertensive groups. During the tetraethylammonium effect the cardiac index showed little change and in some cases rose to a level above that of the control value.

In the sedated hypertensive group there was little fall in the cardiac index during the tetraethylammonium period in spite of marked falls in the blood pressure. In no case in the whole series did the cardiac index fall below the normal limit during the tetraethylammonium effect.

In both normotensive and hypertensive patients a sympathicolytic agent such as tetraethylammonium may produce a fall in blood pressure without significant fall in cardiac index.

# AMINO ACID EXCRETION IN DEGENERATIVE DISEASES OF THE NERVOUS SYSTEM

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THE recent investigations of Uzman and Denny-Brown<sup>1</sup> and Cooper and co-workers<sup>2</sup> have shown excessive urinary amino acid excretion to be a consistent feature of hepatolenticular degeneration even in early and mild states of the disease. Occurrence of this amino-aciduria with only minimal evidence of general liver dysfunction suggests that it may represent the result of a specific metabolic defect and that such defect may have a direct relationship to cerebral degeneration. It therefore seemed of interest to study amino acid excretion in a group of patients with other syndromes characterized by chronic neuronal degeneration, particularly those of familial type.

A second type of metabolic abnormality in Wilson's disease is indicated by the marked increase in copper content of both brain and liver noted by Haurowitz,<sup>3</sup> Glazebrook,<sup>4</sup> and in the well-controlled observations of Cumings.<sup>5</sup> Mandelbrote and associates<sup>6</sup> reported high urinary copper output in a single case of Wilson's disease and found that in this condition, as in normal persons, the administration of BAL (2, 3-dimercaptopropanol) greatly increased copper excretion. BAL might thus provide a tool for the demonstration of a possible relation between these two established biochemical abnormalities. The effect on the amino-aciduria of a marked, though transient, disturbance of copper metabolism as produced by BAL was therefore investigated.

## MATERIAL AND METHODS

Amino acid excretion was studied in representative cases of Huntington's chorea, paralysis agitans, dystonia musculorum deformans, familial spastic paraplegia associated with mental defect, and hepatolenticular degeneration. Urine samples were collected over a known time interval, usually between two and three hours. In all female subjects, and in male subjects uncooperative because of intellectual impairment, collection was by in-lying catheter. The total fasting urinary  $\alpha$ -amino acid nitrogen excretion in milligrams per hour was determined by the ninhydrin-carbon dioxide method of Van Slyke, MacFadyen, and Hamilton.<sup>7</sup> Analyses for tryptophane were carried out by the *p*-dimethylaminobenzaldehyde method of Bates,<sup>8</sup> after mercuric sulfate precipitation as described by Folin and Ciocalteu.<sup>9</sup> Xanthurenic acid was tested for by the color reaction with ferric chloride reported by Lepkovsky and co-workers.<sup>10</sup> Chromatographic analyses for the presence of unusually large amounts of individual amino acids were performed by the method of Dent<sup>11</sup> using the one-dimensional technique with phenol as the solvent. In patients with hepatolenticular

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degeneration, copper was estimated by the diethyldithiocarbamate method of Eden and Green.<sup>12</sup> Acid-cleaned glass and water redistilled over glass were used throughout in the collections and analyses for copper. Blank determinations were performed to correct for minute amounts of copper in the reagents.

## RESULTS

With the exception of hepatolenticular degeneration, none of the conditions studied showed a significant elevation in urinary total  $\alpha$ -amino acid nitrogen excretion per hour when compared with control values on eight individuals which averaged 4.7 mg. per hour, range 3.5 to 6.8 mg. per hour (Table I). Excretion of tryptophane and xanthurenic acid did not differ grossly from normal with the relatively crude methods employed. Chromatographic studies gave no indication of excessive excretion of any one amino acid. The usual finding by this method was two distinct spots with  $R_f^*$  values of about 0.34 and 0.51, identified by superposition with knowns as glycine and alanine. As in normal urine, a third faint spot with an  $R_f$  value of about 0.83 also was present occasionally.

TABLE I. TOTAL URINARY  $\alpha$ -AMINO NITROGEN IN DEGENERATIVE DISEASES OF THE CENTRAL NERVOUS SYSTEM

PATIENT	$\alpha$ -N (MG. PER HR.)	PATIENT	$\alpha$ -N (MG. PER HR.)
Huntington's chorea		Dystonia musculorum deformans	
M. H.	4.4	E. S.	3.2
	4.0		2.3
			7.9
R. G.	3.8	W. McC.	3.6
C. W.	5.2		
	4.4	Familial spastic paraplegia	
T. B.	3.5	W. McD.	3.3
	3.7		3.6
Paralysis agitans		J. J.	4.1
A. H.	4.9		4.0
	4.1	Hepatolenticular degeneration	
L. F.	3.0	A. G.	19.4
	3.6		16.5
		E. R.	11.0
		M. R.	12.0
			13.7

The average value of 14.5 mg.  $\alpha$ -amino nitrogen per hour in the three cases of hepatolenticular degeneration confirms the marked increase in urinary total  $\alpha$ -amino nitrogen excretion previously observed in this disease by Uzman and Denny-Brown. The absence of such a finding in the other conditions studied suggests that  $\alpha$ -amino nitrogen determination may be of value in the differential diagnosis between Wilson's disease and other diseases which occasionally give similar clinical pictures.

The initial urinary copper values of 23.5 and 11.0  $\gamma$  per hour indicate a definitely increased copper excretion in hepatolenticular degeneration when

\* $R_f$  indicates the ratio of the distance along the filter paper moved by the amino acid to the total distance traveled by the solvent.

compared with control figures on four individuals averaging 5.3  $\gamma$  per hour, range 3.0 to 7.9  $\gamma$  per hour. These controls are in good agreement with the more extensive observations of Mandelbrote and co-workers<sup>6</sup> who reported a normal average of 4.85  $\gamma$  per hour, range 2.1 to 9.0  $\gamma$  per hour.

Administration of BAL sufficient to increase the copper excretion by more than seven times the pre-BAL figure did not reduce the amino-aciduria of hepatolenticular degeneration to the control range (Table II). In the first patient injection of BAL was followed by a reaction characterized by profuse sweating, congestion of the sclera, puffiness of the face, nausea, vomiting of about 100 c.c. of bright red blood, and almost complete anuria which persisted for about one-half hour. Intravenous saline infusion was started as soon as the severity of the reaction became evident. It is possible that the apparent drop in amino acid excretion in the first period immediately after BAL may be in part accounted for by this transient extreme oliguria. Subjective improvement, diminished tremor, and increased activity about the ward were noted in this patient following BAL, but it is possible that these changes did not exceed the range of natural fluctuation in the disease. This improvement has persisted during the five months of observation since BAL administration. In the second patient the dose of BAL was smaller, reaction was minimal, and urine volume was maintained by constant intravenous saline infusion throughout the experiment. Amino acid output under these conditions showed no significant change. Subjectively and objectively the clinical status of this patient remained unaltered.

TABLE II. THE EFFECT OF BAL ON AMINO-ACIDURIA IN HEPATOLENTICULAR DEGENERATION

TIME OF COLLECTION PERIOD (MIN.)	URINE VOLUME (ML. PER HR.)	$\alpha$ -AMINO N (MG. PER HR.)	URINE COPPER ( $\gamma$ PER HR.)
Patient A. G.: Received BAL 7 mg. per Kg. intramuscularly			
90 min. before BAL	213	16.5	23
BAL administered			
0-125 min. after BAL	143	10.6	181
125-190	495	20.8	133
190-357	439	19.0	61
357-482	322	17.9	40
Patient E. R.: Received BAL 4 mg. per Kg. intramuscularly			
125 min. before BAL	85	11.0	11
BAL administered			
0-69 min. after BAL	76.5	11.7	101
69-131	136	14.5	87
131-256	190	12.3	26

It is necessary to emphasize that the small number of observations must limit conclusions from the data here reported. Further, chromatography and the methods used for estimating tryptophane and xanthurenic acid were employed only as screening procedures to detect changes of marked degree. However, they did serve to diminish the likelihood that alteration in excretion of individual amino acids was occurring without change in the total  $\alpha$ -amino nitrogen. The method used for determination of total  $\alpha$ -amino nitrogen, on the other hand, is one of great precision which appears to exclude increase in total amino acid excretion in Huntington's chorea, paralysis agitans, dystonia musculorum deformans, and familial spastic paraplegia and to differentiate these

degeneration, copper was estimated by the diethyldithiocarbamate method of Eden and Green.<sup>12</sup> Acid-cleaned glass and water redistilled over glass were used throughout in the collections and analyses for copper. Blank determinations were performed to correct for minute amounts of copper in the reagents.

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The initial urinary copper values of 23.5 and 11.0  $\gamma$  per hour indicate a definitely increased copper excretion in hepatolenticular degeneration when

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## PARENTERAL NUTRITION

### X. OBSERVATIONS ON THE USE OF A FAT EMULSION FOR INTRAVENOUS NUTRITION IN MAN

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**A**N EMULSION of fat consisting of 15 per cent coconut oil, 4.3 per cent dextrose, and a combination of 0.5 per cent soybean phosphatides and 1 per cent polyglycerol esters as stabilizers was reported by this laboratory to have been administered successfully to animals (rats, cats, rabbits, and dogs) and to man.<sup>1</sup> The size of the fat particles of this emulsion was below 1  $\mu$  in diameter for the most part and none were larger than 3 microns. The preparation of this emulsion has been fully described in earlier papers.<sup>2, 3, 4</sup> While this emulsion (referred to as Emulsion 41 in the earlier report<sup>1</sup>) has repeatedly been used with success in man, it was pointed out<sup>1</sup> that it was not completely satisfactory from the clinical viewpoint because after a period of approximately one month it developed pyrogenic properties. The purpose of this paper is to present additional information on the use of Emulsion 41 in man, although neither the cause nor prevention of the slow development of pyrogenic activity<sup>1</sup> is yet clearly understood. However, these studies were all carried out with batches of emulsion less than four weeks of age and, hence, within the time period that it may be used clinically without pyrogenic reaction.

#### EXPERIMENTAL

The emulsion used in the present study was of the composition of Emulsion 41<sup>1</sup> and was prepared in this laboratory. Before clinical use, the sterility of each batch of emulsion was determined and the following routine tests were done: (a) rapid injection (15 ml. per kilogram body weight) intravenously into three adult rats daily for seven days and subsequent post-mortem examination, (b) rapid injection (15 to 20 ml. per minute) intravenously into one adult dog for two successive days in an amount of 5 Gm. of fat per kilogram of body weight, and (c) rapid injection (10 ml. per kilogram body weight) intravenously into adult rabbits for pyrogenic assay. Every batch of emulsion had to pass satisfactorily all of these tests before it was used in the clinic. On the average, an emulsion had been prepared for approximately a week before it was used in man. Plastic disposable infusion sets were employed, and an infusion rate of 4 to 5 ml. per minute was used in adults and 1 to 3 ml. per minute in children, depending on the weight of the child. Viscosity of the emulsions was such that a No. 26 needle could be employed when necessary.

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TABLE I. SUMMARY OF INTRAVENOUS ADMINISTRATION OF A 15 PER CENT FAT EMULSION (EMULSION 41) TO A VARIETY OF PATIENTS  
(EACH 100 ML. OF EMULSION PROVIDES APPROXIMATELY 160 CALORIES)

PATIENT AND DIAGNOSIS	DAILY QUANTITY OF FAT GIVEN I.V.	RATE OF INFUSION (ML./MIN.)	DAYS INFUSION GIVEN	REACTION OF PATIENT
Pt. 1 (4B708), 37 kg., 54 yr., W., F.: Anorexia and weight loss due to intractable epigastric pain	500 ml.	4-5	10	None
Pt. 2 (4B529), 40 kg., 24 yr., W., M.: Ileostomy for ulcerative colitis	600-900 ml.	4-5	10	None
Pt. 3 (H9129), 60 kg., 73 yr., W., M.: Carcinoma of the rectum with metastases	300-400 ml.	4-5	10	None
Pt. 4 (H8718), 40 kg., 55 yr., W., F.: Rheumatoid arthritis and diarrhea of 5 mo. duration	500 ml.	4-5	8	None
Pt. 5 (3A198), 55 kg., 65 yr., W., M.: Comatose following brain operation for metastases of hypernephroma	300-500 ml.	3	12	If more than 0.8 Gm. fat/kg. body weight given, increase in respiratory rate and temperature elevation of 1 to 3°
Pt. 6 (7A320), 37 kg., 35 yr., W., F.: Bronchiectasis with malnutrition	300-900 ml.	4-5	6	None
Pt. 7 (P1153), 45 kg., 68 yr., W., F.: Ovarian carcinoma with metastases	300 ml.	4-5	3	None
Pt. 8 (9A857), 41 kg., 32 yr., W. F.: Chronic nephritis, uremia, hypertension, and pericarditis	300 ml.	3	3	None
Pt. 9 (311320), 11 kg., 4 yr., W., F.: Mental defect with malnutrition	100-300 ml.	2	6	If more than 3.5 Gm. fat/kg. body weight given, temperature elevation of 1 to 2°
Pt. 10 (A341898), 3.5 kg., 7 wk., W., M.: Postoperative obstruction	50-150 ml.	1-2	11	None
Pt. 11 (341528), 15 kg, 6½ yr., W., M.: Ruptured appendix, peritonitis, and multiple abscesses	300-500 ml.	3	27	If more than 4 Gm. fat/kg. body weight given, temperature elevation of 1 to 2°

#### CLINICAL OBSERVATIONS

Table I summarizes the data on the administration of fat emulsion to patients on the surgical and medical services of the Peter Bent Brigham Hospital and the Children's Hospital. The cases represent a wide variety of common ailments which result in weight loss of such an extent that provision for adequate calories becomes one of the major facets of good treatment (Table I). Only those patients who presented unusual findings with regard to the administration of the fat emulsion will be discussed and this discussion will be limited to that aspect of the cases.

*Patient 1.*—This patient was a 54-year-old, single, white woman weighing 37 kilograms admitted to the surgical service with the presenting complaint of anorexia and weight loss due to intractable epigastric pain aggravated by ingestion of food. There was a history of abdominal pain for thirty-three years and a total of eight abdominal operations. The patient appeared poorly nourished. On initial administration of fat, 300 ml. of emulsion were given in a period of sixty minutes without reaction. Thereafter, and for the next

three days, 500 ml. of emulsion furnishing approximately 800 calories were given daily with no subjective complaints or objective findings.

Before and during the administration of fat, observation of this patient's dietary habits revealed that she was a finicky eater and consumed an average of only about 1,200 calories per day. It was assumed that on such a caloric intake, this patient would be in negative nitrogen balance and would continue to lose weight, but that when this oral caloric intake was supplemented with 800 calories coming from fat emulsion the patient ought to be in positive nitrogen balance. This hypothesis was tested by doing a nitrogen and potassium balance study. The protein content of the 1,200 calorie oral diet was 45 Gm. and was provided mostly by animal protein. The potassium content of the daily diet

### EFFECT OF INTRAVENOUS FAT ON NITROGEN AND POTASSIUM BALANCE

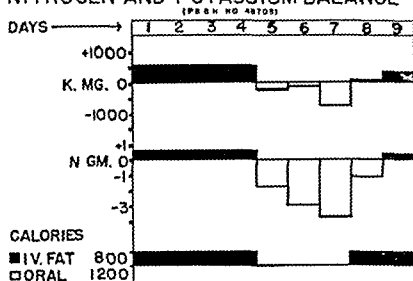


Fig. 1.

was 1.6 grams. Nitrogen determinations were all done by a macro-Kjeldahl method, and potassium was determined by the cobaltinitrite precipitation method.\* Due to circumstances beyond our control, the periods with and without supplementary calories from the fat emulsion were not as long as desired. The results of this study are shown in Fig. 1. It is seen that during the first four-day period when the patient was receiving a total of 2,000 calories (1,200 calories orally which represented the average of the total daily caloric intake during hospitalization and 800 calories from fat given by vein) a positive potassium and nitrogen balance was obtained. During the three-day period when fat infusions were not given and the total caloric intake was only 1,200 calories, the patient promptly went into strong negative balance in both potassium and nitrogen. When fat infusions again were given, positive balances were obtained again. The nitrogen and potassium contributed by the emulsion were negligible.

**Patient 2.**—This patient was a 24-year-old white man weighing 40 kilograms with ulcerative colitis. An ileostomy had been done but did not function properly and the patient developed a peritoneal infection and a midjejunal fistula. It was necessary to reoperate to close the fistula. For several days prior to surgery, the patient's oral intake was nil and the parenteral therapy of blood, 5 per cent glucose, and electrolyte solution contributed no more than 400 calories per day. Because of small, sclerotic veins, 10 per cent glucose and protein hydrolysate preparations were not used. On the first postoperative day, 300 ml. of the fat emulsion were given. For ten of the next twelve days, daily infusions of fat emulsion varying from 600 to 900 ml. which furnished 960 to 1,440 calories were given. An exceptionally favorable clinical response followed the administration of the fat. At the end of this time oral intake of food was sufficient and all parenteral therapy was

\*We are indebted to the Department of Surgery, Peter Bent Brigham Hospital, for doing the potassium determinations.



terminated. The caloric intake of this patient for the immediate postoperative period is shown in Fig. 2. It is seen that during the first three postoperative days, the fat emulsion contributed appreciably to the caloric intake; in fact, during the second and third postoperative days it furnished approximately 75 per cent of the total caloric intake. It was during this critical period that the favorable clinical response was especially noted. Because Emulsion 41 was not available, none was given on the fourth and fifth days. The data in Fig. 2 also emphasize quite strikingly the importance of ordinary food as a source of calories.

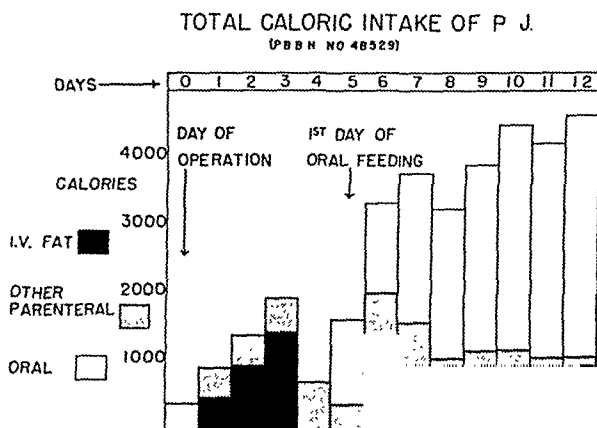


Fig. 2.

*Patient 9.*—This patient was a mentally defective child, 4 years of age, weighing 11 kilograms. Fat emulsion was given on six successive days in amounts varying from 100 to 300 ml., which furnished 30 to 60 per cent of the total calorie requirements. It was observed consistently that infusions of more than 250 ml. (3.5 Gm. fat per kilogram) at any one time produced an elevation of temperature of 1 to 2° which persisted for one to three hours following the fat infusion.

*Patient 10.*—This patient was a 7-week-old male infant, weighing 3 kilograms, with postoperative obstruction, who was on continuous gastric suction and complete parenteral feeding. The theoretical caloric requirement for this infant was approximately 300 calories and the usual parenteral fluids available could provide only approximately a third of this requirement. He was given fat emulsion by vein for eleven days, during which time he received on some occasions as much as 70 per cent of his basal caloric requirements from this source. Fig. 3 illustrates the caloric intake of this infant during a twelve-day period, the last nine days of which fat emulsion was given. Since the two preliminary small infusions of fat were well tolerated, the study presented in Fig. 3 was begun. The fat infusions were increased gradually in amount until by the third day they contributed more than one-half the total caloric intake (Fig. 3). On the fourth day and for the next five days, the theoretical caloric requirement of the infant was more than met, and calories from the emulsified fat furnished approximately two-thirds of the total caloric intake. Throughout this period the child was quite ill as a result of an extensive infection. Antibiotic therapy was ineffective and early in the morning of the thirteenth day of this study the child expired. Post-mortem studies gave no evidence that the extensive fat infusions, daily for nine days and on two other days shortly before the period represented by Fig. 3, had in any way contributed to the death.

*Patient 11.*—This patient was a 6½-year-old boy, weighing 15 kilograms, with a ruptured appendix, peritonitis, and multiple abscesses in whom complete parenteral nutrition was necessary. He received fat emulsion intravenously for twenty-seven days in quantities providing him with 480 to 800 calories per day which provided 50 per cent or more of his

daily calorie requirement. It was observed regularly in this patient that if more than 400 ml. of fat emulsion were given at any one time, there was a gradual rise of temperature of 1 to 2° during one to three hours following the infusion. This quantity of emulsion contained 60 Gm. of fat which furnished this patient with 4 Gm. of fat per kilogram. The infusions of fat in this child were clinically effective in that he presented a problem of complete parenteral feeding and further weight loss was prevented during the period fat infusions were given.

### TOTAL CALORIC INTAKE OF S.E.

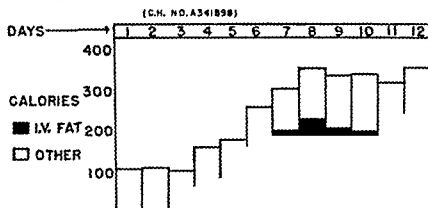


Fig. 3.

In view of the fact that an increase in temperature of 1 to 2° was consistently obtained in Patient 11 when the amount of fat given exceeded 4 Gm. per kilogram body weight, it was thought desirable to attempt to determine which component of the emulsion was largely responsible for the temperature rise. The following three different preparations were studied in Patient 11: the usual 15 per cent fat emulsion (Emulsion 41); a 15 per cent emulsion identical to Emulsion 41 except that the costabilizer of polyglycerol esters was reduced from 1 per cent concentration to 0.4 per cent; a fat-free emulsion which contained only the two stabilizers in the same concentration as in Emulsion 41 in 5 per cent dextrose. A different one of these three preparations was infused in Patient 11 in the same amount on each of three successive days. For the next three successive days these preparations were given in the same order, but the one without fat was given in twice the amount. The temperature rise occurred only when the amount of fat-containing emulsion infused exceeded 4 Gm. fat per kilogram of body weight.

That this temperature rise following infusion of a fat emulsion varies considerably among patients is suggested by the observations in Patient 9 in whom an amount exceeding 3.5 Gm. fat per kilogram of body weight resulted in a rise in temperature and in Patients 2 and 10 in whom as much as 3 and 6 Gm. fat per kilogram respectively did not give rise to an increase in temperature, yet it was the same batch of fat emulsion which was given to Patient 11. The data in Table I show that Patient 5, a 65-year-old man, weighing 55 kilograms, with cerebral metastases from a hypernephroma, and in whom most of the left frontal lobe had been removed, developed a temperature rise if more than 300 ml. of fat emulsion were given at any one time. This amounts to only 0.8 Gm. of fat per kilogram of body weight.

Insufficient evidence is available to indicate whether this rise in temperature is due to the amount of fat per se or to pyrogens contained in the fat which, when they reach a certain concentration, give rise to the increase in temperature. At present, the latter seems the more likely cause.

## DISCUSSION

The clinical observations presented in this paper indicate that fat emulsions as a source of calories in parenteral nutrition are useful in a wide variety of cases. Emulsion 41 furnished 1,600 calories per liter, and could be infused at the rates commonly employed for the intravenous administration of glucose or saline solutions.

Previous studies in the dog and rat have shown conclusively that such fat emulsions of fine particle size introduced directly into the systemic circulation are utilized for energy purposes as indicated indirectly by total carcass analysis for fat,<sup>5</sup> maintenance of normal weight,<sup>5</sup> conversion of negative to positive nitrogen balance on a low-protein low-calorie ration,<sup>5</sup> and growth of puppies,<sup>6</sup> and directly by radioisotope studies showing that  $C^{14}$  introduced as part of the fat molecule in a fat emulsion is rapidly eliminated in the expired  $CO_2$ .<sup>7</sup> In previous studies with experimental animals and man, it has been shown that fat emulsions such as have been employed in the studies can be given safely and without producing pathologic conditions.<sup>1, 3</sup>

The studies reported in this paper extend many of these observations in man. The observations on Patient 1 confirm the favorable effect of fat emulsions administered intravenously on nitrogen balance. The similar observations on potassium balance further emphasize the necessity of adequate calories for protein synthesis in the body. The very favorable clinical response observed in the patient severely ill with ulcerative colitis (Patient 2) is attributed in part to the calories supplied by the fat emulsion during the first three postoperative days. In Patients 10 and 11, fat emulsions were given for rather long periods, eleven and twenty-seven days respectively, and in generous daily amounts, approximating 6 and 4 Gm. fat per kilogram of body weight. Patient 10, who was severely ill from a generalized infection, expired after nine successive days of fat infusion given in such an amount that calories from fat furnished two-thirds of the total caloric intake for a period of seven days. However, at autopsy, there was no abnormal accumulation of fat in any of the organs and no evidence that the fat infusions in any way had contributed to the death. During this severe illness, when all nourishment had to be given by vein, weight loss was prevented.

An increase in temperature in Patients 5, 9, and 11 was regularly observed following a single administration of a certain amount of fat. However, the amount of fat necessary to cause this temperature rise varied widely among these three patients—from 0.8 to 4 Gm. fat per kilogram of body weight. Patient 2, who received 3 Gm. fat per kilogram of body weight, and Patient 10, who received 6 Gm. fat per kilogram of body weight, showed no increase in temperature. It is thought that these temperature rises were due to pyrogenic materials in the fat and not to the fat per se.

Of the patients listed in Table I, three died by the time of the preparation of this paper—Patients 5, 8, and 10. Post-mortem examinations were obtained on the latter two and in neither was there any gross or microscopic pathologic condition attributable to the fat emulsions. Patient 5 expired approximately

one month following the last fat infusion; Patient 8, three months following the last infusion; and Patient 10, eighteen hours following the last infusion of fat.

It should be pointed out that the reason more fat emulsion was not given to many of these patients, and for longer periods of time, was that not enough was available because of limited production and control facilities and the needs of various experimental studies with animals. As stated in a previous paper<sup>1</sup> on this subject, Emulsion 41 is still not considered completely suitable for clinical use because of the development of pyrogenicity after it is approximately four weeks old.

#### SUMMARY

Observations are reported on the intravenous administration of a 15 per cent fat emulsion to eleven patients representing a variety of common illnesses. Of these patients, eight were adults, two were children, and one was a 7-week-old infant. The emulsion furnished 1,600 calories per liter and was given at rates ordinarily used for administering glucose or saline solutions. Daily amounts up to 3 Gm. fat per kilogram of body weight were given to adults and 6 Gm. fat per kilogram of body weight in a 7-week-old infant. Infusion periods ranged from three to twenty-seven consecutive days. The emulsion was effective as indicated by favorable clinical response, the prevention of weight loss, and the maintenance of positive nitrogen and potassium balance. Subsequent post-mortem examination of three of these patients revealed that the fat emulsions had produced no pathologic changes, either gross or microscopic.

The authors wish to express appreciation to the Medical and Surgical Services of the Peter Bent Brigham Hospital and the Children's Hospital of Boston for their genuine cooperation in these studies, and to The Upjohn Company, Kalamazoo, Mich., which has supplied us generously with various materials used in this research.

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# THE ABSORPTION AND DISPOSITION OF ORALLY ADMINISTERED $I^{131}$ -LABELED NEUTRAL FAT IN MAN

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NONE of the available methods for measuring the absorption and utilization of fat in man is greatly informative and, at the same time, accurate and simple to carry out. The direct chemical determinations of serum fat in serial fashion are tedious and time consuming; in the hands of all save the most expert they are subject to considerable inaccuracies. Relatively large samples are required. No information as to the disposition of the fat is available from this method, i.e., what proportions of the lipid which has disappeared from the blood stream are accounted for by degradation and by storage respectively. The vitamin A tolerance test obviates the first two of these objections, but not the third. Nephelometric techniques for fat analyses are now generally conceded to be unreliable.

The problem had been quite satisfactorily solved for animal work by Geyer and co-workers,<sup>1</sup> and Lerner and co-workers,<sup>2</sup> by the use of neutral fat containing radioactive  $C^{14}$ -substituted fatty acids. However, the extremely long half-life of this isotope precludes its use in man. In addition, other isotopes which emit radiation of greater penetrating power are more simple to measure.

Certain of the shortcomings mentioned may be overcome by the use of neutral fat which has been labeled with radioiodine  $I^{131}$  in the unsaturated fatty acids. It is the purpose of this communication to report tracer studies with such radioactive fat on eighteen patients, including ten normal subjects.

## METHODS

Commercial olive oil was iodinated with  $I^{131}$ .<sup>\*</sup> The following steps were employed. To the carrier-free  $I^{131}$  solution (usually 10 mc.), 10 mg. of sodium iodide were added as carrier. The solution was then placed in a separatory funnel and acidified with 1 ml. of a 50 per cent concentrated nitric and sulfuric acid mixture. The liberated iodine was extracted with chloroform. Into the chloroform solution a stream of chlorine was then passed until the purple color of iodine just disappeared. The iodine chloride obtained was added to a chloroform solution of 20 Gm. of olive oil. This mixture was allowed to stand for twenty-four hours. The chloroform solution then was shaken repeatedly with a solution of sodium carbonate in order to remove any free iodine. The chloroform solution was dried over anhydrous sodium sulfate and the chloroform evaporated off by means of an infrared lamp. The odor, taste, color, and consistency of the oil were unchanged following iodination.

One-half to 5 ml. of the oil, containing 100 microcuries of  $I^{131}$ , were soaked into bread and eaten, either during or immediately following a normal breakfast. In a few instances the patients were without breakfast; all ate a normal mixed diet during the balance of the test period.

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\*By Mr. Charles Margnetti of Tracerlab, Inc., Boston, Mass.

The radioactivity in the thyroid gland was estimated by serial counting at a distance of 35 cm. from the neck with a sensitive, directionally shielded gamma tube (Sylvania). The "background" (from extrathyroidal tissues plus cosmic and other extraneous radiation) was calculated by counting over the lower thigh; this was subtracted from the thyroid count. The resulting net count was compared with that obtained under similar geometric conditions from a suitable aliquot of the original solution.

One milliliter portions of urine in uniform glass vials were counted with the gamma tube through an orifice in the shield and compared with equal volumes of suitable dilutions of the original solution. Since the bottoms of the vials were in contact with the metal tube surface, excellent reproducibility and sensitivity (272 net counts per second per microcurie) were obtained.

Two-tenth milliliter aliquots of serum were pipetted onto flamed, 1 inch copper planchets, each of which was partially covered with a smaller disk of thin absorbent paper. Since the planchets were grease-free and were coated with cupric oxide, the liquid was absorbed uniformly into the paper. After slow drying with an infrared lamp, the serum formed an evenly adherent film, the area of which was limited to that of the paper circle. The preparations were permanently mounted by covering with Scotch tape. Standards were prepared by evaporating suitable aliquots of petroleum ether solutions of the original material in the same manner; self-absorption was equalized by adding identical amounts of serum. The samples were counted with a thin-window Geiger-Müller tube.

Two milliliters of serum were diluted, precipitated with zinc sulfate, and the excess zinc precipitated with alkali by the method of Somogyi. Five milliliter portions of the supernatant (equivalent to 0.5 ml. serum) were evaporated onto disks and the radioactivity was compared with similarly prepared standards as described. Since the lipids were precipitated with the proteins, the radioactivity in the watery fraction of the serum represented inorganic iodine which had been removed from the fat. By subtraction of this "water-soluble" portion from the total, a fraction designated as the "lipid  $I^{131}$ " was obtained.

In seven patients the sera were separated by means of Bloor's solution and the radioactivity was determined in the lipid and water-soluble fractions of this extract. There was, in general, agreement between the results obtained by this procedure and the former one. However, there was usually slightly more radioactivity in the portion resulting from petroleum ether extraction of the dried Bloor's extract than when this lipid fraction was calculated by subtraction. Because of the greater simplicity of the zinc precipitation method, it was used exclusively later.

## RESULTS

In ten normal subjects the pattern of absorption and disposition was constant. The concentrations of total  $I^{131}$  in the serum were greatest at three to six hours following the meal and slowly decreased after this time. The peaks in both the "total  $I^{131}$ " and "lipid  $I^{131}$ " curves occurred simultaneously (Fig. 1). These levels of total radioactivity ranged from 0.4 per cent to 0.6 per cent per 100 ml. of serum, whereas the highest concentrations of the "lipid" fractions varied from 0.20 per cent to 0.47 per cent per 100 ml. of serum.

The accumulation of radioactivity by the thyroid gland varied from 10 to 25 per cent in twenty-four hours (Fig. 1). Although appreciable, this was somewhat less than the average collection of a dose of inorganic  $I^{131}$  administered for the purpose of ascertaining thyroid function.<sup>3</sup> Likewise, the excretion in the urine of 15 to 48 per cent of the ingested radioiodine by the normal controls in twenty-four hours was distinctly less than if the material administered had been inorganic iodine.

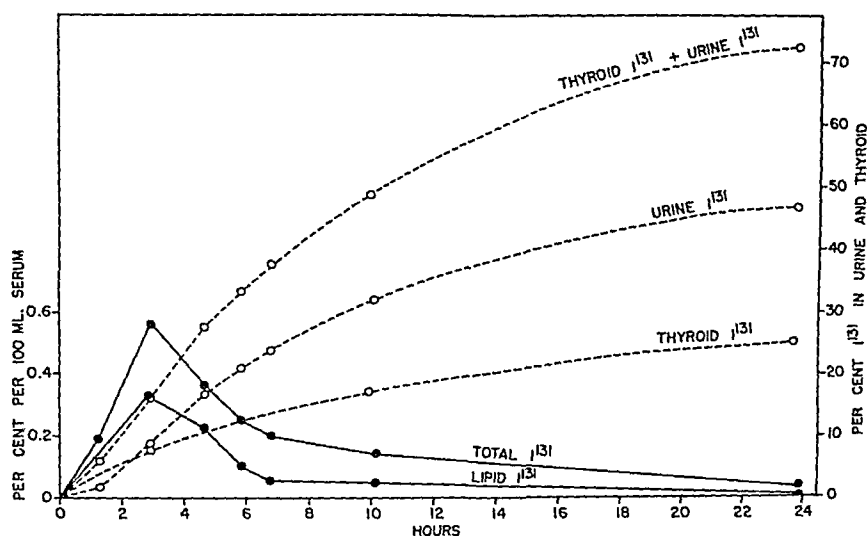


Fig. 1.—Curves showing the fate in a normal subject of radioiodine introduced into the body as labeled fat. The time in hours following the ingestion of the iodinated fat is plotted on the abscissa. Along the left ordinate are plotted the concentrations of radioactivity attained on the serum; variations in these values are indicated by the solid lines. The broken lines show the percentage of administered radioactivity which was excreted in the urine and collected by the thyroid (right vertical scale).

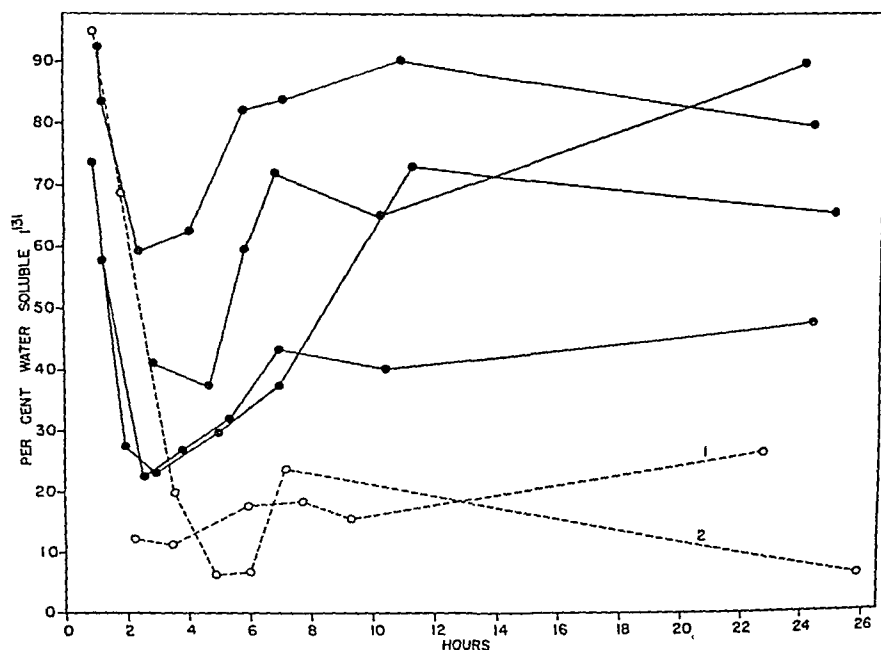


Fig. 2.—The variation with time of the fraction of radioactivity in the "water soluble" portion of the serum. The sera were fractionated as described in the text; the "water-soluble  $^{131}$ " is plotted as per cent of the total radioactivity. The solid lines and circles show these values in four normal subjects while the broken lines and open circles denote a patient with idiopathic hyperlipemia (2) and the nephrotic syndrome (1). In the normal subjects (and in the patient with idiopathic hyperlipemia [2]) during the first hour of the test 60 to 90 per cent of the radioactivity was in the water-soluble fraction. In the normal subjects this sharply decreased during the next one to two hours, as absorption of the iodinated olive oil reached a maximum, and returned slowly to the high values during the next four to seven hours. In the hyperlipemic patients the percentage of radioactivity in the water-soluble portion of the serum remained low throughout the rest of the test after the first two hours.

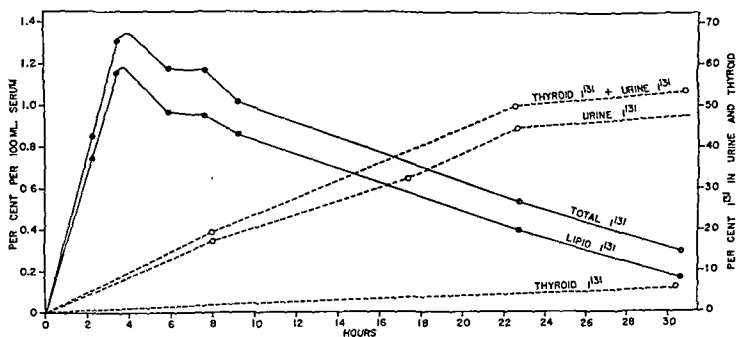


Fig. 3.—The absorption and disposition of labeled fat in a patient with the nephrotic syndrome. The data are indicated as in Fig. 1. It will be seen that the concentrations of radioactivity in the serum, principally in the lipid fraction, are much greater than normal; the decrease in these high levels is abnormally slow. The excretion of  $I^{131}$  in the urine and its accumulation in the thyroid, especially during the early hours of the test, are also somewhat slower than normal.

The changes in the ratio of serum inorganic to "lipid" iodine also were uniform (Fig. 2). During the first one to two hours from 60 to 90 per cent of the  $I^{131}$  was in the water-soluble portion. Later, as absorption of the iodinated olive oil progressed the size of the inorganic fraction decreased sharply, then gradually returned to the high values during the ensuing eighteen hours.

In three patients with high serum neutral fat levels (two subjects with "idiopathic" hyperlipemia and one individual with the nephrotic syndrome) the peak "lipid  $I^{131}$ " values were two to five times normal.<sup>4</sup> There was an abnormally slow decrease in the radioactivity during the twenty-four hours following these high values. A larger than normal portion of the radioiodine remained in the lipid fraction (Fig. 2), and the accumulation of radioactivity in the thyroid and the excretion of the radioiodine in the urine were abnormally slow also (Fig. 3). In one patient with hypercholesterolemic xanthomatosis the test was normal. In one case of sprue the highest serum concentrations of total  $I^{131}$  were about one-half normal. The urinary excretion and thyroid collection were correspondingly low, but there was a normal relationship between the inorganic and "lipid" fractions in the serum at all times.

#### DISCUSSION

It should be emphasized that the procedure under discussion was not a "tolerance test" in the usual meaning of the term. The amount of oil administered was never more than 5 Gm.; this served to label the fat being absorbed from the meal eaten with the tracer, as well as that already in the blood stream.

Olive oil contains about 80 per cent glycerol trioleate and about 7 per cent trilinoleate. The former of these 18-carbon fatty acids possesses one double bond between the 9th and 10th carbon atoms, while the latter has two such



bonds. The saturated compounds tristearate and tripalmitate comprise the bulk of the remainder of the lipids of the oil.

As a result of iodination the radioiodine was firmly attached at the double bonds in the unsaturated fatty acid chains; the process used was similar to the familiar method for determination of the "iodine number" of fats. In order to be certain that this iodine was organically bound, petroleum ether solutions of the oil in each lot were repeatedly extracted with water; no radioactivity was detectable in the aqueous phase.

It may be accepted as proved that the iodine collected by the thyroid gland is in the form of the iodide ion.<sup>5</sup> The identity of the iodine excreted in the urine by the subjects in this study has not been determined, although it is entirely likely that it is in the same form. The radioactivity in the water-soluble portion of the serum and that collected by the thyroid and excreted in the urine represent iodine which has been removed from the fatty acid skeleton during the process of metabolism; these fractions provide an index of the rate of utilization of the iodinated fat.

In this study, from 50 to 73 per cent of the administered radioactivity was collected in the thyroid gland and excreted in the urine of normal subjects during the twenty-four hours from the beginning of the test, with approximately one-half these amounts accounted for during the first six hours. These figures indicate the extent of degradation of the fatty acid skeleton of ingested neutral fat which normally occurs in men, either as a preliminary to storage in the fat depots or, much more likely, for production of energy. The characteristic appearance of an appreciable portion of radioactivity in the water-soluble fraction of the serum during the early hours of the test suggests that a small amount of breakdown may have occurred during the process of absorption from the gastrointestinal tract.

That a similarly rapid metabolism of intravenously administered neutral fat takes place in the rat has been recently demonstrated by Geyer and co-workers<sup>1</sup> and by Lerner and associates.<sup>2</sup> The latter group used a 16-carbon fatty acid (as tripalmitin) with the 6th carbon atom labeled with C<sup>14</sup>; as much as 59 per cent of the injected C<sup>14</sup> was found in the expired air in twenty-four hours. The type of fat administered and the position of the labeled atom were not identical in the experiment of Lerner and co-workers and in the present study. However, the curves representing the sum of urinary excretion plus thyroid accumulation of I<sup>131</sup> in the normal subjects in this study closely resemble those depicting the cumulative excretion of C<sup>14</sup>O<sub>2</sub> by the rats in the experiment of Lerner, Chaikoff, Entenman, and Dauben.

#### SUMMARY

Characteristic curves of absorption and utilization of physiologic amounts of lipid taken by mouth by subjects on a mixed diet were demonstrated by the use of unsaturated fats iodinated with I<sup>131</sup>. The proportion of the administered radioactive iodine which was collected by the thyroid and excreted in the urine, as well as the magnitude of the water-soluble portion in the serum, indicated

the extent of breakdown of the labeled fat. Under these circumstances in normal subjects, degradation of from 50 to 73 per cent of the orally administered iodinated fat took place within twenty-four hours. Subjects with "idiopathic" hyperlipemia and the nephrotic syndrome utilized the labeled lipid much more slowly.

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# THE EFFECT OF SPLENECTOMY ON THE TOXICITY OF $\text{Sr}^{90}$ TO THE HEMATOPOIETIC SYSTEM OF MICE

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## INTRODUCTION

**R**ADIOSTRONTIUM ( $\text{Sr}^{90}$ ), a  $\beta$ -ray emitter with a fifty-five day half-life, which is physiologically interchangeable with calcium, localizes largely in bone soon after enteral or parenteral administration.<sup>1-6</sup> The minimum intraperitoneally administered dose required to produce a significant leucopenia in CF-1 female mice<sup>6</sup> was found by Simmons and Jacobson<sup>7</sup> to be about 0.068 microcuries per gram of body weight. However, this leucopenia was relatively transient. Brues and co-workers<sup>8</sup> have reported that bone sarcomas are readily produced in this strain with this dose. Although a severe and persistent leucopenia developed after an intraperitoneal dose of 2.0 microcuries per gram of body weight, no significant anemia occurred.<sup>7</sup> In a preliminary experiment<sup>9</sup> histopathologic examination of mice sacrificed at intervals after injection revealed much depleted or aplastic bone marrow, whereas the spleens contained a remarkable increase in erythrocytopoiesis. The fact that anemia failed to develop under these circumstances was considered to be directly related to the rapid development of an intense ectopic erythrocytopoiesis in the spleens of the radiostrontium-treated animals. Accordingly, an experiment of greater scope, as described in this communication, was undertaken to determine the validity of this concept.

## MATERIALS AND METHODS

Young CF-1 female mice were divided into three groups of fifty or more animals each as shown in Table I. The mice in Group I were splenectomized; those in Group II received a single intraperitoneal injection of 2.0 microcuries of  $\text{Sr}^{90}$  per gram of body weight, and the mice in Group III were splenectomized and later injected with a dose of 2.0 microcuries per gram of body weight. A fourth group of animals not included in Table I, which was neither splenectomized nor given  $\text{Sr}^{90}$ , was used for base line hematologic controls and from this group animals were sacrificed at intervals to serve as normal reference for the histologic studies. Splenectomies were performed in Groups I and III while the animals were under ether anesthesia. Twenty-four days after splenectomy,  $\text{Sr}^{90}$  was administered intraperitoneally to the animals in Group III. The average weight of the mice at the time of injection was 20 grams. A second injection of 2.0 microcuries per gram was given to a representative number of animals in Groups II and III, 119 days after the original injection. Although a leucopenia of moderate degree persisted at this time in both groups (II and III), the animals in Group II which had been subjected to splenectomy and received  $\text{Sr}^{90}$  also had

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The photomicrographs were made by Jean M. Crunelle of the Photographic Department, Billings Hospital, University of Chicago.

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\*Raised by Carworth Farms; homozygous for aa bb cc.

TABLE I. GENERAL SCHEMATA OF EXPERIMENT

GROUP	NUMBER OF ANIMALS	TREATMENT	PERIOD OF OBSERVATION AFTER ORIGINAL $\text{Sr}^{90}$ INJECTION
I	51	Splenectomy only	282 days
II*	63	Injected intraperitoneally with $\text{Sr}^{90}$ in a dose of 2.0 microcuries per gram body weight	282 days
III	70	Splenectomy plus $\text{Sr}^{90}$ (2.0 microcuries per gram body weight)	282 days

\*Selected animals in Groups II and III were reinjected with a dose of 2.0 microcuries per gram body weight 110 days after original injection.

recovered from the anemia. The purpose of the second injection of  $\text{Sr}^{90}$  was largely to determine whether or not compensatory ectopic erythrocytopoiesis was sufficient in the splenectomized mice to prevent or minimize recurrence of anemia.

*Preparation of Radiostrontium ( $\text{Sr}^{90}$ ): Distribution and Excretion Studies.*—As has been described elsewhere,<sup>5</sup> the solution of radiostrontium was composed of  $\text{Sr}^{90}$  and  $\text{Sr}^{90}$ .  $\text{Sr}^{90}$  has a fifty-five day half-life and a maximum energy of 1.7 mev;  $\text{Sr}^{90}$ , which represented 3 to 5 per cent of the initial stock solution on arrival from Oak Ridge, has a half-life of circa thirty years and a maximum energy of 0.65 mev. The final preparation for administration was strontium chloride in isotonic solution. The greatest part of the radiostrontium excretion occurred during the first three days, and after ten to fifteen days virtually no more of the retained  $\text{Sr}^{90}$  was excreted. The average total excretion values were 54 per cent of the injected dose. Average total retention values were 45 per cent of the injected dose. By the third day after injection almost all of the retained  $\text{Sr}^{90}$  was deposited in the skeleton. Retention of  $\text{Sr}^{90}$  in the spleen was as follows:

First 24 hours	0.22 per cent/gram
24 to 72 hours	0.04 per cent/gram
3 days to 3 months	0.02 per cent/gram

In other soft tissues of the body, the amount of radiostrontium per gram remaining after three days was well under 0.1 per cent of the injected dose.

*Hematologic Studies.*—Studies of the peripheral blood were made on all four groups. These studies included determinations of the hemoglobin in grams per 100 ml. (photoelectric), erythrocytes and leucocytes per cubic millimeter, leucocyte differentials (Wright's stain), and reticulocytes per cubic millimeter and in per cent (brilliant cresyl blue). Blood for study was drawn from the tail vein.

Control hematologic determinations were made on all animals after which splenectomy was performed on animals in Group I and III. Twenty-four days after splenectomy,  $\text{Sr}^{90}$  was injected intraperitoneally into the animals in Groups II and III. Repeat hematologic determinations were made on all animals seven days after the original  $\text{Sr}^{90}$  injection and thereafter at twenty-one day intervals. After the second injection of  $\text{Sr}^{90}$  in selected animals, hematologic studies were made at less frequent intervals.

*Histologic Studies.*—Animals prepared as described were sacrificed at intervals through 280 days after  $\text{Sr}^{90}$  injection for histopathologic study. In addition, random animals in all groups on which hematologic studies were being conducted were sacrificed at various intervals. This latter procedure was followed in an attempt to correlate more closely the peripheral blood studies with the actual histopathologic state of the blood-forming tissue.

Tissues taken for study included skin, lung, small intestine (with Peyer's patch), adrenal, kidney, liver, spleen, thymus, lymph node, and bone marrow (femur and vertebrae). The tissues were fixed in Zenker-formol, embedded in 20 per cent nitrocellulose, sectioned at 8  $\mu$ , and stained with hematoxylin-eosin-azure II.

## RESULTS

*Hematologic Studies on the Peripheral Blood.—*

(a) *Effect of Splenectomy Alone:* The mean leucocyte value in the peripheral blood of splenectomized mice rose after splenectomy to stabilize between 14,000 and 22,000 per cubic millimeter during the period of observation (Fig. 3). Splenectomy alone reduced the hemoglobin and erythrocyte values slightly during the first three weeks after splenectomy, but thereafter, as is shown in Figs. 1 and 2, no appreciable fluctuation occurred.

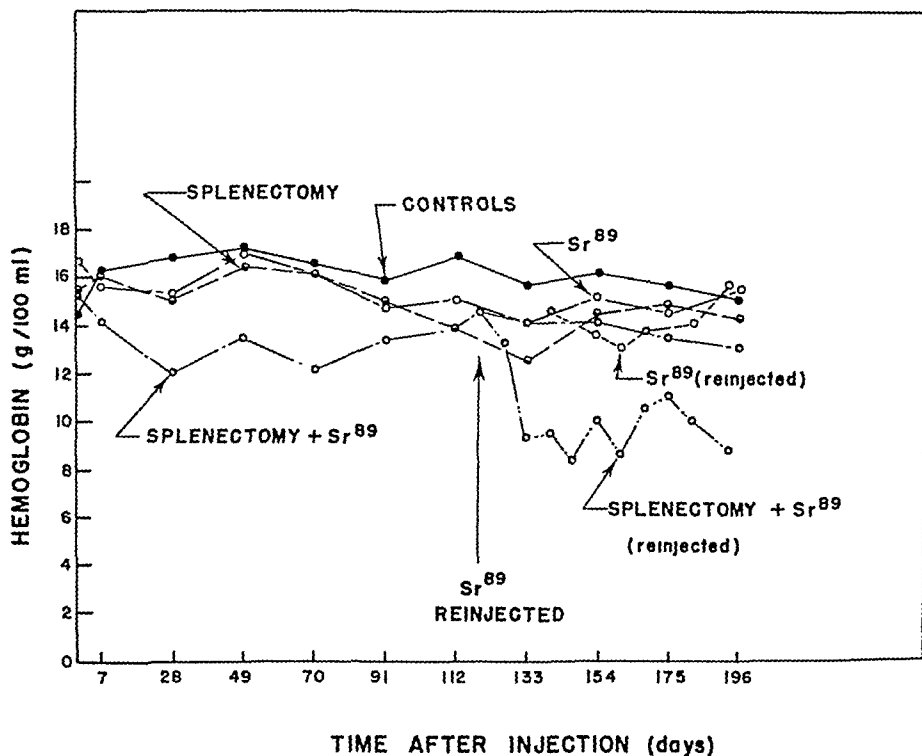


Fig. 1.—The effect of a single injection of 2.0 microcuries of radiostrontium per gram body weight on hemoglobin values of normal mice and mice splenectomized twenty-four days prior to  $\text{Sr}^{89}$ . One hundred nineteen days after the original  $\text{Sr}^{89}$  injection, a second injection of 2 microcuries per gram body weight was given to selected animals from the two groups which originally were injected with this isotope.

(b) *Effect of  $\text{Sr}^{89}$  Injection on Leucocytes of Peripheral Blood:* The intraperitoneal injection of 2.0 microcuries of  $\text{Sr}^{89}$  per gram produced a comparable degree of leucopenia in animals with an intact spleen and in animals that previously had a splenectomy (Fig. 5). As is shown in Figs. 4 and 5, the initial injection of  $\text{Sr}^{89}$  reduced heterophils and lymphocyte values by 52 and 36 per cent, respectively, in twenty-eight days. The mean heterophil value of  $\text{Sr}^{89}$ -injected animals which had intact spleens returned to the preinjection level in about forty-nine days and thereafter was actually higher than the

control value. The mean heterophil value of animals which had been splenectomized and were given  $\text{Sr}^{89}$  returned to the normal control or preinjection level in seventy days. The lymphocyte reduction after  $\text{Sr}^{89}$  injection was sustained in the animals with intact spleens and in splenectomized animals. In animals to which a second injection of  $\text{Sr}^{89}$  was given 119 days after the first injection, the reduction in heterophil and lymphocyte values was greater and more persistent in the splenectomized mice than in the intact mice (Figs. 4 and 5).

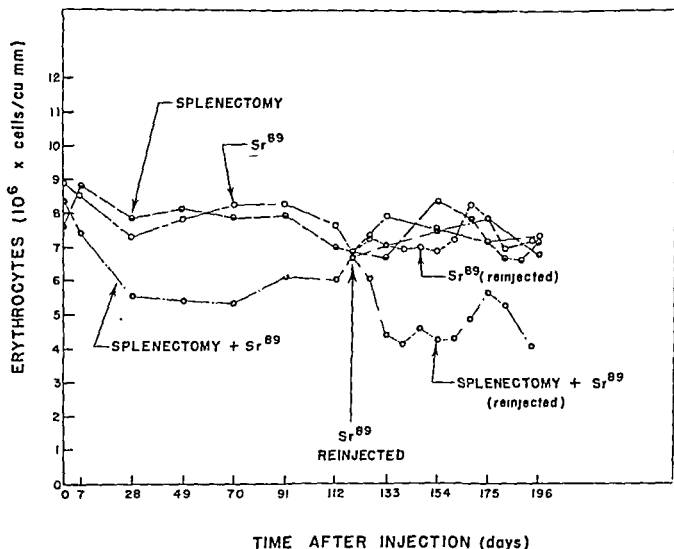


Fig. 2.—The effect of a single injection of 2.0 microcuries of radiostrontium per gram body weight on erythrocyte values of normal mice and mice splenectomized twenty-four prior to  $\text{Sr}^{89}$ . One hundred nineteen days after the original  $\text{Sr}^{89}$  injection, a second injection of 2 microcuries per gram body weight was given to selected animals from the two groups which originally were injected with this isotope.

(c) *Effect of  $\text{Sr}^{89}$  Injection on the Hemoglobin and Erythrocyte Values of the Peripheral Blood:* No anemia of significance was produced in mice with intact spleens to which  $\text{Sr}^{89}$  was given by intraperitoneal injection. On the other hand, splenectomized mice injected with 2.0 microcuries per gram developed a significant anemia in twenty-eight days which persisted through seventy days, but with recovery in 119 days (Figs. 1 and 2). Selected animals with an intact spleen and animals which had been splenectomized were given a second injection of  $\text{Sr}^{89}$  (2.0 microcuries per gram) 119 days after the

original injection. As after the initial  $\text{Sr}^{89}$  injection, the mice with intact spleens developed no anemia, whereas the splenectomized group developed a precipitous reduction in both hemoglobin and erythrocyte values. Although recovery from the anemia proceeded in the splenectomized group, the anemia recurred between the fifty-sixth and seventy-seventh day after the second injection at which time all the remaining animals died or were sacrificed for study (Figs. 1 and 2).

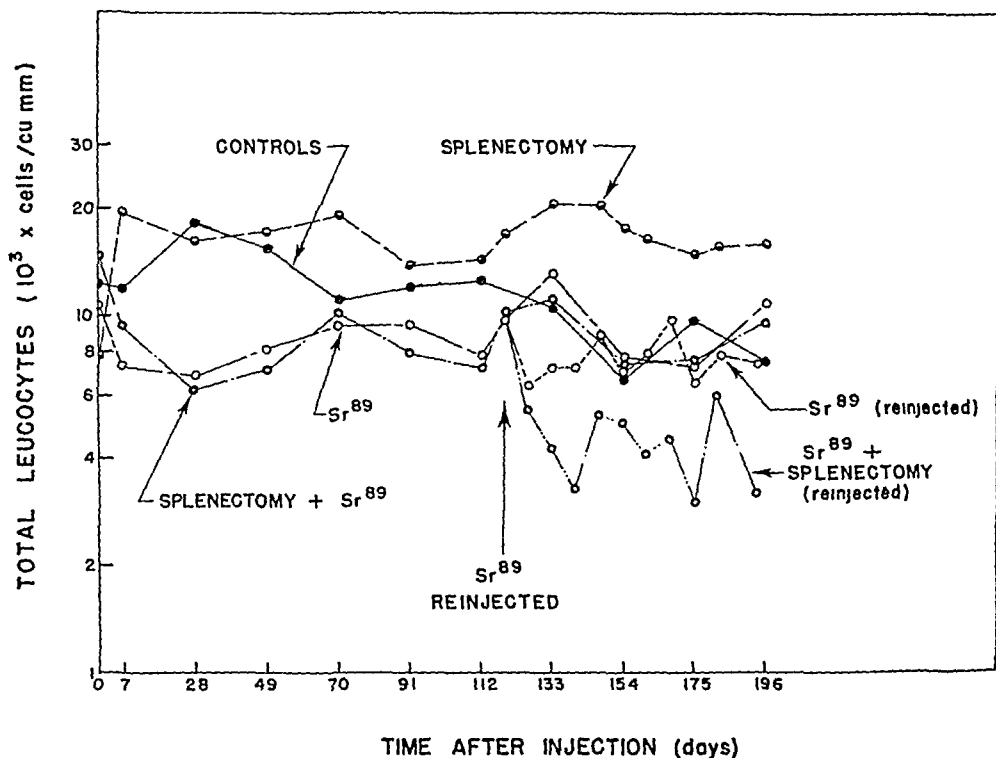


Fig. 3.—The effect of a single injection of 2.0 microcuries of radiostrontium per gram body weight on leucocyte values of normal mice and mice splenectomized twenty-four days prior to  $\text{Sr}^{89}$ . One hundred nineteen days after the original  $\text{Sr}^{89}$  injection, a second injection of 2 microcuries per gram body weight was given to selected animals from the two groups which originally were injected with this isotope.

(d) *Effect of  $\text{Sr}^{89}$  on Reticulocyte Values of the Peripheral Blood:* Except for control studies no reticulocyte determinations were made until seven days after  $\text{Sr}^{89}$  injection; but at this point the reticulocyte values of animals which were splenectomized only and those which were given  $\text{Sr}^{89}$  only had actually risen, whereas the reticulocyte value of the group which was both splenectomized and strontium-treated had fallen significantly. Re-injection of  $\text{Sr}^{89}$  at 119 days produced no reduction in the reticulocyte value of mice with intact spleens. A reduction comparable with that seen after the original injection occurred; however, this was limited to splenectomized mice. As will be evident in the section on histology which follows, more frequent determinations

of the reticulocyte values of all the groups would have been of importance from the point of view of correlation.

### Histopathologic Studies.—

(a) *Observations on Lymphatic Tissue After Single Injection of  $\text{Sr}^{89}$ :* No consistent difference in size of the spleens existed between  $\text{Sr}^{89}$ -injected and control mice which were sacrificed during the 280-day period. However, an effect was observed in both the white and red pulp of the spleen in animals sacrificed three days after  $\text{Sr}^{89}$  treatment. This early effect consisted of a decreased cellularity of the white pulp and a marked hyperplasia of ectopic

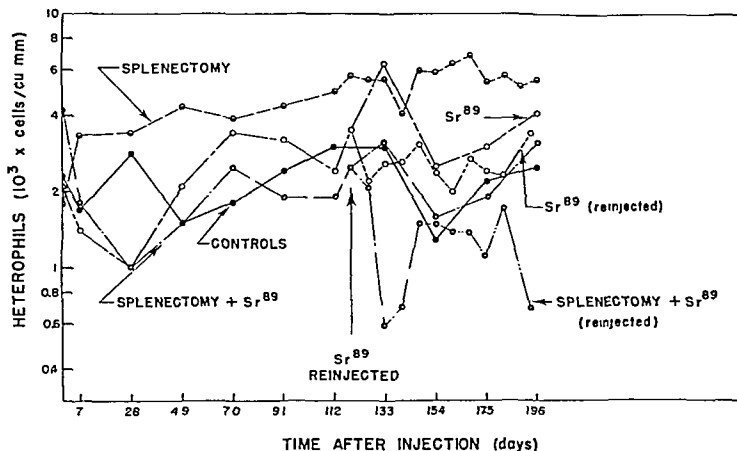


Fig. 4.—The effect of a single injection of 2.0 microcuries of radiostrontium per gram body weight on heterophil values of normal mice and mice splenectomized twenty-four days prior to  $\text{Sr}^{89}$ . One hundred nineteen days after the original  $\text{Sr}^{89}$  injection, second injection of 2 microcuries per gram body weight was given to selected animals from the two groups which originally were injected with this isotope.

erythrocytopoiesis and megakaryocytopoiesis in the red pulp; ectopic granulocytopoiesis was practically absent at this stage. Only a few scattered mature granulocytes could be found. There was little evidence of destructive effects, however, for there was no observable increase in pigment or debris-filled macrophages or pyknotic nuclei at this stage as compared with the spleens of control mice. The spleens of control mice usually contained small foci of ectopic erythrocytopoiesis, granulocytopoiesis, and megakaryocytopoiesis scattered about in the red pulp. The erythrocytopoiesis in the spleens of the  $\text{Sr}^{89}$ -injected animals, however, was diffuse and intense at three days and consisted of predominantly large hemocytoblasts, basophilic erythroblasts, and a lesser number of more mature forms.



The number of young megakaryocytes in the spleens of  $\text{Sr}^{89}$ -injected mice three days after injection was greatly above that of the controls. This intense erythrocytopoiesis and megakaryocytopoiesis persisted in the  $\text{Sr}^{89}$ -injected animals at all the periods of examination through 280 days. Photomicrographs of the spleens of  $\text{Sr}^{89}$ -injected and control mice at intervals of three, twenty-nine, forty-five, and ninety-two days (Fig. 6) illustrate especially the

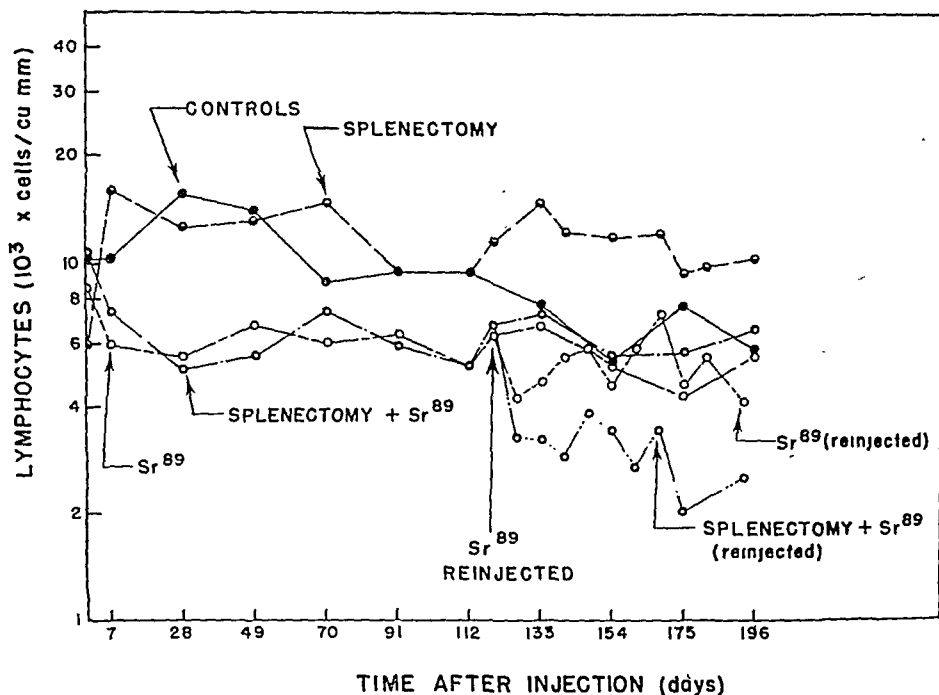


Fig. 5.—The effect of a single injection of 2.0 microcuries of radiostrontium per gram body weight on lymphocyte values of normal mice and mice splenectomized twenty-four days prior to  $\text{Sr}^{89}$ . One hundred nineteen days after the original  $\text{Sr}^{89}$  injection, a second injection of 2 microcuries per gram body weight was given to selected animals from the two groups which originally were injected with this isotope.

degree of erythrocytopoiesis at these stages. Fig. 7 illustrates the closely packed erythroblasts in the red pulp at some of these stages at a slightly greater magnification.

No increase in ectopic granulocytopoiesis was observed in spleens of mice sacrificed at intervals of three and eight days after injection. Increased granulocytopoiesis became apparent in twelve days and increased to a maximum between forty-five and ninety-two days. In fact, in several of the mice examined during the forty-fifth and ninety-second days, granulocytopoiesis was qualitatively as intense as erythrocytopoiesis.

Changes in the white pulp in the spleens of  $\text{Sr}^{89}$ -treated mice were more or less universal at all stages studied from 3 to 280 days after injection. In normal mice the white pulp occupies circa 50 per cent of the spleen and, except for the distinct lymphatic nodules, blends gradually into the red pulp.

The splenic white pulp of the  $\text{Sr}^{90}$ -injected mice almost without exception occupied less space than the red pulp and was distinctly separated from the intense erythrocytopoietic and granulocytopoietic red pulp by a zone of relative acellularity consisting largely of reticular cells (Figs. 6 and 7). The decrease in cellularity of the white pulp was observed especially in the early

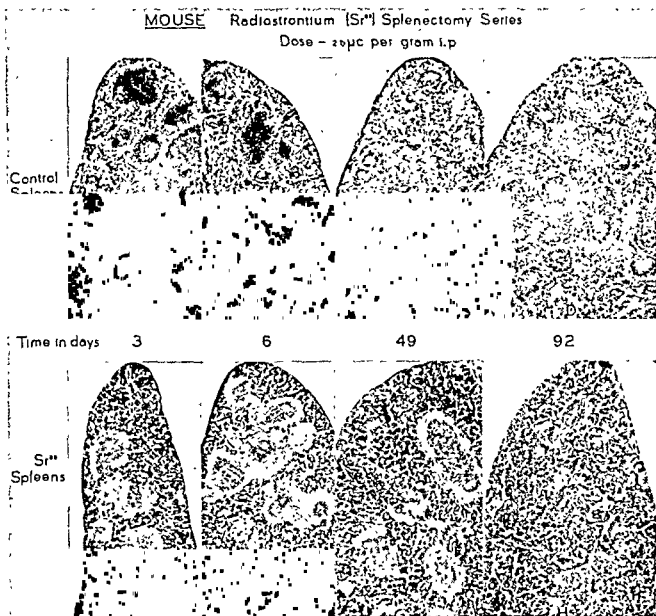


Fig. 6—Photomicrographs of sections of the spleens of mice at intervals after  $\text{Sr}^{90}$  injection. The normal spleens show small areas of ectopic hematopoiesis in the red pulp and the normal variation in the white pulp, whereas the spleens of  $\text{Sr}^{90}$ -injected animals show an intense ectopic hematopoiesis in the red pulp and a depletion of the cellularity of the white pulp. (Hematoxylin-eosin-azure II,  $\times 15$ .)

stages after injection (i.e., three, six, twelve, fifteen, twenty-one, and twenty-nine days), but the atrophy, which persisted, is illustrated in the photomicrograph of the ninety-second day specimen (Fig. 7).

(b) *Lymph Nodes, Peyer's Patches of Intestine, and Thymus:* Lymph nodes from the root of the mesentery and Peyer's patches from the small intestine from all animals sacrificed were studied in control and  $\text{Sr}^{90}$ -injected groups. As opposed to the white pulp of the spleen of  $\text{Sr}^{90}$ -injected animals in which atrophy, reduced cellularity, and absence or diminution in the number of active lymphatic germinal centers were observed, the lymph nodes and

MOUSE Radiostrotrium ( $Sr^{90}$ ) Splenectomy Series  
Dose - 2000  $\mu$ c per gram i.p.

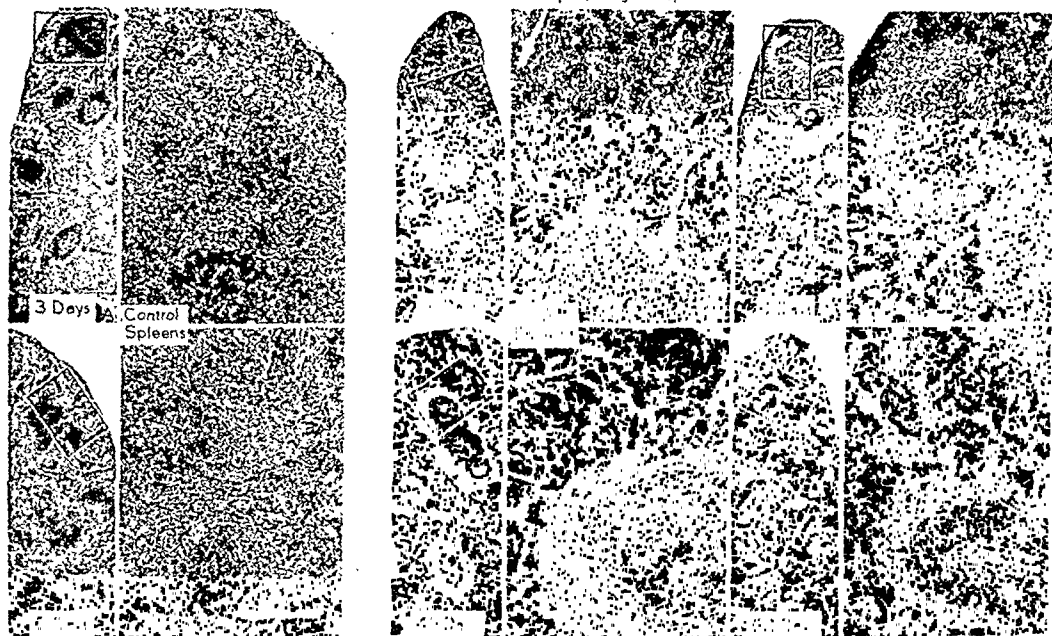


Fig. 7.—Photomicrographs of normal spleens and the spleens of  $Sr^{90}$ -injected animals with selected areas at a higher power to illustrate the intensity of the ectopic hematopoiesis in the red pulp and the depletion of cellularity in the white pulp of  $Sr^{90}$ -injected animals. (Hematoxylin-eosin-azure II; low power  $\times 15$ , high power  $\times 70$ .)

Peyer's patches appeared essentially normal and the germinal centers were active. Granulocytopoiesis, erythrocytopoiesis, and megakaryocytopoiesis were definitely increased in the medulla of the lymph nodes of some of the  $Sr^{90}$ -injected mice as compared with control mice. This increase in ectopic hematopoiesis in lymph nodes was observed as early as fifteen days after injection but was more prominent in later stages. No increase or decrease in plasma cells in lymphatic tissue was observed. No histologic changes which could be directly attributed to  $Sr^{90}$  were observed in the thymus.

(c) *Observations on Lymphatic Tissue After Second Injection of  $Sr^{90}$  at 119 Days:* Only a relatively few stages were studied. These were at fifteen, twenty-four, and seventy-seven days after the second injection.

The histologic effects as far as lymphatic tissues were concerned were again limited to the spleen. The lymph nodes, Peyer's patches, and the thymus appeared essentially normal except for a moderate increase in granulocytopoiesis, megakaryocytopoiesis, and erythrocytopoiesis in the lymph nodes. At the time of the second injection, the erythrocyte and hemoglobin values were essentially normal in both groups of mice which had originally received  $Sr^{90}$  (Figs. 1 and 2). The marked ectopic erythrocytopoiesis, megakaryocytopoiesis, and granulocytopoiesis, however, persisted in the spleens of the  $Sr^{90}$ -injected animals sacrificed at 119 days at a time comparable with

that at which selected animals received a second  $\text{Sr}^{90}$  injection. Although moderate atrophy of the white pulp was also apparent at this time, active lymphatic germinal centers were present and, in general, the white pulp was more cellular than in earlier stages.

By the fifteenth day following the second injection of  $\text{Sr}^{90}$ , ectopic erythrocytopoiesis, megakaryocytopoiesis, and granulocytopoiesis were more intense than at the 119-day stage. Ectopic granulocytopoiesis, however, was only moderate in degree. Again, as after the first injection of  $\text{Sr}^{90}$ , little evidence existed of active cellular disintegration in the form of pyknosis, karyorrhexis, or phagocytosis. The white pulp, which showed recovery to some extent after the original injection of  $\text{Sr}^{90}$  at 92 and 119 days in that it constituted a more normal percentage of the splenic tissue and was richer in medium and small lymphocytes, again was reduced in amount with a depletion of lymphocytes in the periphery of the white pulp fifteen days after the second injection. In the two other stages studied following the second injection, namely twenty-four and seventy-seven days, the recovery of the white pulp was only moderate; atrophy and decreased cellularity persisted.

As was true after the initial injection of  $\text{Sr}^{90}$ , essentially "maximum" increased ectopic erythrocytopoiesis and megakaryocytopoiesis were apparent fifteen days after the second; granulocytopoiesis was progressively more intense at the twenty-four and seventy-seven day stages. Pigment-filled macrophages, scattered throughout the red pulp, were increased in number at all stages studied after the second injection.

(d) *Observations on Bone Marrow After a Single  $\text{Sr}^{90}$  Injection:* The observations made on bone and bone marrow are limited to the effects of  $\text{Sr}^{90}$  on hematopoiesis in the marrow spaces. A vertebra and a femur were taken for study from each animal sacrificed. Since the effects of  $\text{Sr}^{90}$  on vertebral or femoral marrow were essentially comparable in the mice with intact spleens and in those that were splenectomized prior to the injection, no attempt to differentiate between the two groups will be made in the description of marrow changes. In general, the destructive effects of  $\text{Sr}^{90}$  on hematopoiesis were comparable in femoral and vertebral marrow in that if complete or partial atrophy were found in the entire femur, the condition was also present in the vertebral bone marrow and vice versa. However, moderate decrease in cellularity with or without fibrosis was not infrequently found in the metaphyseal region of the femur immediately adjacent to the epiphyseal cartilage without evidence of such localized changes in the vertebral marrow. The destructive effects of  $\text{Sr}^{90}$  and the subsequent depletion of the normal hematopoietic cells were most severe in the metaphyseal region, of equal or slightly less severity in the epiphyseal marrow, and least in the diaphysis of the femur. In some instances essentially complete depletion of normal hematopoiesis was found in the entire femoral and vertebral marrow spaces of mice sacrificed at fifteen through eighty days. In others, although general cellularity of the femoral diaphysis and vertebral marrow space was reduced, complete depletion was present only in the metaphyseal and epiphyseal regions of the bone.

In spite of the cellular depletion that occurred after  $\text{Sr}^{90}$  injection, no stage studied either in femoral or vertebral marrow showed clear-cut histologic evidence of increased death of cells. The number of pyknotic nuclei and macrophages filled with particulate debris did not exceed that that was observed in control mice. A marked depletion of cells in the epiphyseal and

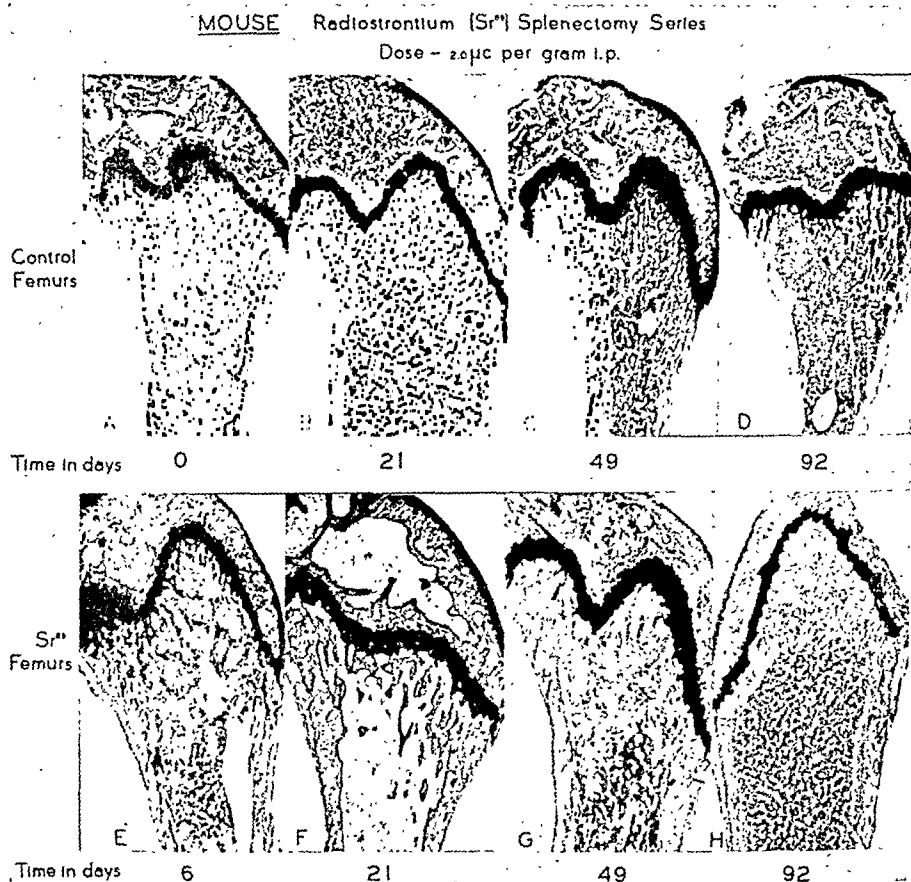


Fig. 8.—Photomicrographs of normal femoral marrow and of femurs of  $\text{Sr}^{90}$ -injected animals at various intervals after the  $\text{Sr}^{90}$  injection. These photomicrographs illustrate the depletion of normal hematopoietic cells in the epiphyseal and metaphyseal regions with partial sparing in the diaphysis of some animals. (Hematoxylin-eosin-azure II,  $\times 15$ .)

metaphyseal marrow space of the femur was present three days after injection. The general cellularity of the diaphysis of the femur as well as in the vertebral marrow space was moderately decreased. In the epiphyseal and metaphyseal regions of the femur only a few scattered heterophils, plasma cells, and large lymphocytes remained at this stage, whereas dilated vascular spaces and increased fat cells filled the area. Distally from the depleted metaphyseal region, the cellularity of the marrow gradually increased. The diaphyseal marrow was diffusely cellular but distinctly less so than that of

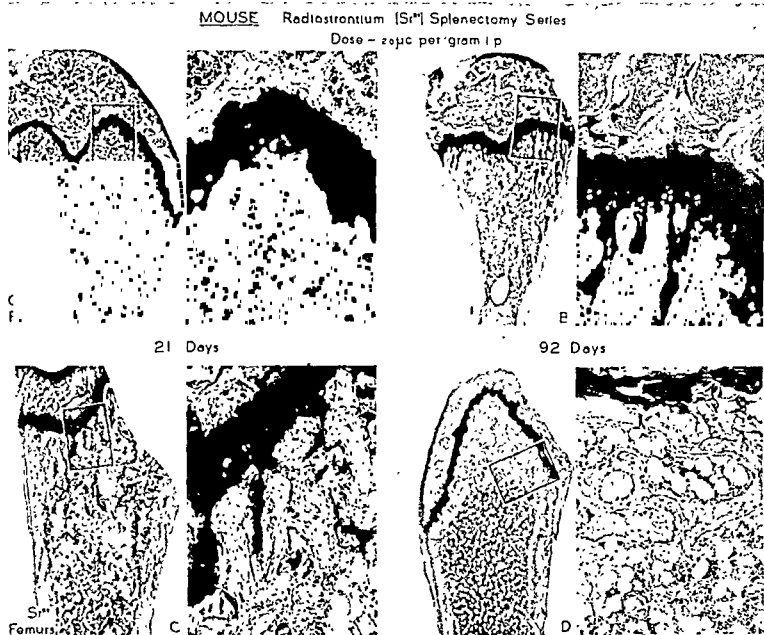


Fig. 9.—Photomicrographs of normal femoral marrow and femurs of  $\text{Sr}^{90}$ -injected animals with selected areas at a higher power to illustrate the loss of normal cellularity and replacement by fibrous tissue (C) twenty-one days after  $\text{Sr}^{90}$  injection and the residual cellular depletion in the metaphysis ninety-two days after  $\text{Sr}^{90}$  injection. (Hematoxylin-eosin-azure II; low power  $\times 15$ , high power  $\times 70$ .)

normal control mice. The vascular spaces in this part of the marrow at three days were conspicuously dilated; megakaryocytes were fewer in number; and erythrocytopoiesis was definitely decreased. The remaining cells were thus largely composed of the precursors of the granulocytic series.

The vertebral marrow was comparable with that of the femoral diaphysis at this stage. At six days the findings were essentially the same as those for the three-day stage (Fig. 8, E). The degree of marrow depletion varied considerably from animal to animal at various time intervals after injection. In Fig. 8, F the femur of a mouse sacrificed twenty-one days after injection is shown. In this specimen only fat and dilated vascular spaces filled with erythrocytes remained. In two other mice sacrificed at this same interval the epiphyseal and metaphyseal regions of the femurs were largely depleted of cells; the diaphyseal portions contained about as many hematopoietic cells as the controls. In still another mouse sacrificed at this interval the vertebral

and femoral marrow contained small areas of normal-appearing hematopoietic cells scattered about among the fat cells, dilated vascular spaces, and gelatinous areas; whereas the metaphyseal portion was more or less completely replaced by fibrous tissue (Fig. 9, C). At later stages, i.e., 49, 71, 92, and 119 days after  $\text{Sr}^{90}$  injection, the depletion of cells in the metaphyseal and epiphyseal portions of the femoral marrow remained conspicuous (Figs. 8, G and 9, D). The shaft or diaphyseal portion of the femur and the vertebral marrow approached more normal hematopoietic activity. Likewise, in these stages the amount of fat and the dilation of the vascular spaces became less conspicuous.

(c) *Observations on Bone Marrow After Second  $\text{Sr}^{90}$  Injection:* Selected animals were given a second injection of  $\text{Sr}^{90}$  119 days after the first injection. At this time (Figs. 1 and 2) no anemia existed in either group which had originally received  $\text{Sr}^{90}$ . Residual partial cellular depletion or fibrosis in the epiphyseal and metaphyseal region of the femur still persisted at this stage, but the diaphyseal portion of the femur and the vertebral marrow were only slightly less cellular than in normal mice. Sacrifices of mice that were given a second  $\text{Sr}^{90}$  injection were made fifteen, twenty-four, and seventy-seven days after the injection. The effect on the marrow of these mice was essentially the same as that observed in comparable stages after the first injection except that the number of pigment-filled macrophages was slightly increased in the reinjected animals.

(f) *The Effect of  $\text{Sr}^{90}$  on Other Tissues or Organs:* The tissues or organs, not hitherto discussed, which were studied were skin, lung, adrenal, small intestine, kidney, and liver. No cytologic evidence of destructive effect which could be attributed to  $\text{Sr}^{90}$  was noted in these organs or tissues. In an occasional animal, which had been given a single injection of  $\text{Sr}^{90}$  or reinjection with a comparable dose of  $\text{Sr}^{90}$ , ectopic erythrocytopoiesis was found in the liver and adrenal. In each instance, however, this was minimal.

#### DISCUSSION

In spite of the extensive cellular depletion that develops in the bone marrow of mice injected with  $\text{Sr}^{90}$  in a dose of 2 microcuries per gram of body weight, no anemia develops. Intense ectopic erythrocytopoiesis occurs with such rapidity in the spleens of these mice that the steady state of the circulating erythrocytes remains essentially normal. Splenectomized mice, on the other hand, develop an anemia after the injection of this dose of  $\text{Sr}^{90}$  because of the fact that compensatory ectopic erythrocytopoiesis in the spleen is not possible and its development in other potential sites is not adequate. Increased ectopic megakaryocytopoiesis in the spleens of  $\text{Sr}^{90}$ -injected animals occurred as rapidly as ectopic erythrocytopoiesis, but since platelet determinations on the peripheral blood were not performed, no statement can be made with reference to whether or not this compensation was sufficient to prevent platelet reduction. In experiments suggested by the results related in this communication Jacobson and associates<sup>10</sup> showed that lead protection of the surgically mobilized spleens of mice during the administration of 600 r x-radia-

tion to the balance of the body obviated the development of anemia and significantly reduced the severity and the duration of the leucopenia and thrombocytopenia which regularly follow the delivery of this dose to the whole animal. Histologic studies showed that this phenomenon was due to the rapid development of ectopic blood formation in the lead-protected spleens.

An interesting problem that has been posed by this experiment relates to the fact that lymphocytopoiesis was markedly reduced in the spleens of  $\text{Sr}^{90}$ -injected animals, whereas the lymphatic tissue in the lymph nodes from the root of the mesentery, the thymus, and in Peyer's patches of the small intestine was essentially unaltered at any stage studied. The amount of  $\text{Sr}^{90}$  found in the spleen of injected animals was minimal twenty-four hours after injection and practically none was found three days after injection. It seems unlikely, therefore, that the reduction in lymphatic tissue in the spleen extending for more than ninety days after  $\text{Sr}^{90}$  injection could be attributed to the transient presence of  $\text{Sr}^{90}$  in the spleen. Neither can one explain this reduction in splenic lymphocytopoiesis adequately on the basis of radiation originating from  $\text{Sr}^{90}$  deposited in bone since one would expect other lymphatic tissue (lymph nodes, etc.) to be affected in a comparable manner. It would be difficult to explain the reduction in lymphatic tissue in the spleen on an indirect basis for the same reason. Although differences in the radiosensitivity of lymphatic tissue in various sites throughout the body of the mouse may exist, it seems unlikely that such an explanation is warranted here. The most plausible explanation for the decrease in splenic lymphatic tissue is that it may be related to a nutritional competition between the lymphatic tissue and ectopic hematopoiesis. The compensatory granulocytopoiesis in the spleen of  $\text{Sr}^{90}$ -injected mice reached a maximum at a time approximately comparable with the time at which the heterophils in the peripheral blood returned to normal values (forty-five to seventy days).

The delay in the appearance of ectopic granulocytopoiesis in the spleens of these animals suggests that this process was suppressed either primarily or secondarily, that the stimulus for an intensification of this process was less marked, or that even with a maximum stimulus the spleen had only a capacity for a gradual intensification of ectopic granulocytopoiesis.

The fact that anemia recurred in the splenectomized mice after the administration of a second injection of  $\text{Sr}^{90}$  at 119 days indicates fairly conclusively that ectopic erythrocytopoiesis in sites other than the spleen is wholly inadequate to cope with the destruction and suppression of erythrocytopoiesis which occurs in the bone marrow. The eventual recovery of splenectomized mice from the anemia induced by the first  $\text{Sr}^{90}$  injection must therefore have been largely a function of the recovery of erythrocytopoiesis in the marrow spaces.

The fact that histologic evidence of destruction in the bone marrow and spleen other than cellular depletion and the late appearance of pigment-filled macrophages was not seen in  $\text{Sr}^{90}$ -injected animals should perhaps be com-



mented upon. For example, as early as three days after  $\text{Sr}^{90}$  injection, partially to essentially complete depletion of hematopoietic cells in the bone marrow was seen. Only dilated vascular spaces, fat cells, a few debris-filled macrophages and occasional plasma cells, lymphocyte-like cells, etc., remained in the bone marrow of some of the animals sacrificed at this stage. It is likely that debris-laden macrophages, pyknotic nuclei, etc., would have been more in evidence had specimens been taken earlier than three days. Likewise, in the white pulp of the spleens of  $\text{Sr}^{90}$ -injected animals, active phagocytosis was minimal even though the general cellularity was markedly decreased in three days.

#### SUMMARY AND CONCLUSIONS

Radiosttrontium ( $\text{Sr}^{90}$ ), a  $\beta$ -ray emitter with a half-life of fifty-five days, is largely deposited in the skeleton when administered to the experimental animal. Although deposition in bone is general, the greatest concentration of this isotope invariably occurs in areas of active bone growth.

A persistent leucopenia but no anemia of significance was produced in the peripheral blood of young mice given a single intraperitoneal injection of this isotope as the chloride in a dose of 2.0 microcuries per gram. The same dose produced a leucopenia and an anemia in splenectomized mice. Recovery from the anemia was essentially complete by 119 days after the  $\text{Sr}^{90}$  injection. Hematopoiesis was markedly reduced in the bone marrow of all radiostrontium-treated animals within three days after injection. The epiphyseal and metaphyseal regions of the long bones were largely depleted of cells, or, in some instances, the latter regions were completely replaced by fibrous tissue. Recovery of hematopoiesis in the bone marrow of the femur was essentially complete by 119 days except that some cellular depletion still existed in the metaphysis at this stage.

Ectopic erythrocytopoieses and megakaryocytopoiesis were greatly increased in the spleen three days after  $\text{Sr}^{90}$  injection as was true of all other intervals studied through 119 days. Splenic lymphocytopoiesis, on the other hand, was reduced in three days and recovery was not complete by 119 days. Lymphocytopoiesis was essentially unaffected in other lymphatic tissues (lymph nodes, thymus, etc.). Ectopic granulocytopoiesis increased more slowly in the spleens of  $\text{Sr}^{90}$ -injected animals, reaching a maximum at circa sixty days. Ectopic granulocytopoiesis, megakaryocytopoiesis, and erythrocytopoiesis were not remarkable in other tissues of the animals which received  $\text{Sr}^{90}$ .

The rapid development and the persistence of ectopic erythrocytopoiesis in the spleen prevent anemia in mice given  $\text{Sr}^{90}$  intraperitoneally in a dose of 2.0 microcuries per gram.

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# JAPANESE B ENCEPHALITIS: REPORT OF FIVE CASES

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## INTRODUCTION

THE epidemic of Japanese B encephalitis in Japan in the summer of 1948 was of significant interest because of the opportunity it afforded for direct observations on this exotic disease by physicians of the occupation forces. From a panoramic public health viewpoint, it was essentially of minor import as the actual percentage of the population afflicted was exceedingly small. In view of the rapidly expanding geographical confines of the disease, it may present a diagnostic and therapeutic problem to clinicians in the United States in the near future.

## EPIDEMIOLOGICAL AND ETIOLOGICAL ASPECTS

The purpose of this article is to point out the clinical observations on a small series of five serologically proved cases observed at the Navy Dispensary, Yokosuka, Japan. Nevertheless, it does not seem amiss to discuss some of the features of the epidemiological and etiological aspects.

The geographical distribution of the disease is rather widespread, cases having been reported from Okinawa and the near-by islands, Korea, China, Guam (epidemic of 1947), Siberia, the Japanese islands, and possibly many other countries of the Far East.<sup>1, 2, 3</sup>

*Vectors.*—In our present state of knowledge, it is believed that the encephalitic virus is conveyed by a mosquito. Inada,<sup>4</sup> a Japanese investigator, and two Russian workers, Petrischeva and Shublada,<sup>5</sup> have reported the isolation of the virus from *Culex tritaeniorhynchus* and *Culex pipiens* var. *pallens*. Animal reservoirs of infection have been discovered in horses, pigs, goats, cows, and possibly sparrows, but not in chickens.<sup>1, 2</sup> It is interesting to note, as demonstrated in studies in Japan, that outbreaks among domestic animals may occur coincident with no or few human clinical cases.<sup>6</sup> Presumably epidemic outbreaks may occur only when the factors of mosquitoes, animal reservoirs of infection, and human susceptibles exist concomitantly. The disease has presented a fluctuating course in Japan, reaching epidemic or minor epidemic proportions every few years, a feature probably related to the accumulation of susceptible persons. In July, 1924, there were 6,125 cases with a mortality rate of 62 per cent. In 1935, there was an outbreak of 5,370 cases.<sup>7</sup> One interesting aspect of the epidemic under discussion is that the conditions of temperature and rainfall were exceedingly favorable for the propagation of mosquitoes prior to the outbreak of the disease.

*Incidence in Man.*—Apparently, from evidence accumulated from many sources, the clinically recognizable cases represent only a relatively small

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proportion of the total, the majority smoldering under the blanket of sub-clinical or very mild clinical infection. In 1936, one year after a large epidemic, 83 per cent of 116 sera tested in Tokyo revealed virus neutralizing antibodies.<sup>6</sup> In 1937, 0.8 per cent of 525 human sera obtained from Hokkaido, Japan, demonstrated these antibodies.<sup>6</sup> Hokkaido is not an epidemic nor an endemic area of the disease. In 1941, 83 per cent of 104 sera from Middle China possessed neutralizing ability against the Japanese B virus,<sup>8</sup> and, in 1945, 90 per cent of the natives of Okinawa, similarly, had neutralizing antibodies.<sup>1</sup> In 1946, a similar phenomenon was found in the sera of 85 per cent of thirteen Chinese residents of Shanghai and 89 per cent of nineteen Chinese residents of Tientsin.<sup>3</sup>

Sabin<sup>1</sup> reported a mortality rate for the 1945 outbreak on Okinawa of 28.6 per cent, and on the near-by islands of Ieanya and Hamahika, 30.6 per cent. Among American military forces on Okinawa in 1945, there were two deaths occurring in a series of twelve severe cases. Figures compiled by the Public Health and Welfare Section, Supreme Commander for Allied Powers, revealed that in the first forty-one weeks of 1948 there were approximately 8,000 cases of Japanese B encephalitis throughout the empire, and 2,374 deaths were reported. The approximate mortality rate from the disease was 25.7 per cent for the Tokyo area and 35.7 per cent for all Japan.<sup>7</sup> In the summer of 1948, there were three fatalities in the twenty-nine proved cases of Japanese B encephalitis in occupation personnel in Japan. Because of the paucity of serologically proved cases occurring among occupation personnel in Japan, a study of the features of the disease as manifested in the five cases forming the subject of this paper seemed all the more desirable.

Although superficially the number of deaths in 1948 attributable to the encephalitic virus seems fairly large, it shrinks considerably when viewed relative to the 78 millions inhabiting the Japanese islands. These fatalities fade almost into oblivion when one contemplates the staggering total of approximately 147,000 deaths in Japan in 1947 directly attributable to tuberculosis. According to several attending Japanese physicians, approximately 2 per cent of the patients exhibit sequelae. These include clonic muscular movements, decerebrate rigidity, and twitchings of muscle groups.

The figures for age incidence in the Tokyo area in 1948<sup>7</sup> reveal a high rate for the younger age group, i.e. through 1 to 20 years of age. The highest occurred in the 6 to 10 years group, the rate being 117.3 per 100,000. The lowest incidence occurred in the 31 to 70 years group, the rate being 9.1 per 100,000. In the early years, male patients have the highest incidence of the disease, but this tendency decreases after the age of 20 until there is a female preponderance in the 51 to 60 years group. In all ages, the mortality among female patients is higher than that among male patients. The over-all case fatality rate in Tokyo during the 1948 epidemic was 19.9 per cent for males and 33.3 per cent for females. These figures were derived from 2,067 cases.

Two cases, occurring in nonvaccinated Naval personnel, are of interest in that they assist in elucidating the time requisite for the incubation period of the

disease. Two firemen from a Naval vessel stayed ashore overnight in Yokohama. The ship remained in Yokohama for two days, sailing Aug. 1, 1948. Many mosquitoes were observed aboard ship. On August 11 one man reported to the ship's sick bay with a complaint of headache of four hours' duration. He seemed drowsy and had a fever. The patient was transferred to a British hospital at Ceylon thirty hours later and died twenty-four hours after transfer. Unfortunately, no autopsy was performed. On August 21 the other fireman developed a headache and fever and became disoriented. A spinal fluid examination performed at a hospital at Bahrein Gulf showed a slight pleocytosis and an increase in protein. The patient's clinical course was similar to that of cases of Japanese B encephalitis occurring in Japan. Following complete recovery, the man returned to Japan. His complement-fixing titer one month after the original infection was 1:16, and the neutralization index was 63,000.

*Status of Vaccination.*—The status of vaccine has not been established at this time. No conclusions relative to the efficacy of vaccination could be drawn from the epidemic on Okinawa. Sabin<sup>9</sup> has shown a rise in neutralization and complement-fixing antibodies in young people inoculated with mouse brain vaccine, two 1 c.c. doses being given six days apart and the last dose of 1 c.c. one month later. Very elderly persons who had no previous serologic neutralizing antibodies exhibited no response. Following the administration of a potent vaccine, many persons develop a rise in neutralizing antibodies.<sup>7</sup> In American adults, no rise in complement-fixing antibodies occurs as a result of vaccination alone, although a person previously infected with the live virus may develop complement-fixing antibodies after vaccination.<sup>7</sup> The vaccine used at present is a chick embryo vaccine, and its efficacy is under intense investigation.

Twelve patients of the twenty-nine cases occurring among occupational personnel in Japan had received complete immunization. No deaths occurred among this group. Three patients had received complete immunization except for one dose. No deaths occurred among these. Six patients had failed to obtain complete vaccination by two or more doses. There was one death among them. Two deaths occurred among eight completely nonimmunized patients.<sup>7</sup>

*Symptoms.*—The clinical symptoms of the disease as previously reported by Sabin<sup>1, 2</sup> include marked lethargy, confusion, high fever, nuchal rigidity, leucocytosis, and spinal fluid pleocytosis. The onset is abrupt, with sudden headache and fever. Sabin, in his Okinawa series<sup>1</sup> (ten serologically proved), observed convulsions in only two cases, both of them fatal. He stressed the duration of the fever, seven to eleven days, and the constant bradycardia present. Cells in the spinal fluid were predominantly mononuclear, whereas a polymorphonuclear leucocytosis was found in the peripheral blood smear. One patient of his series exhibited interesting sequelae, i.e., hyperirritability, mask-like facies, somnolence, and a propensity for urinating on the floor. In the Korean outbreak, two instances of bladder paralysis were recorded. One of these patients died. In the other, bladder function became normal upon convalescence.

*Serologic Studies.*—Serologic studies upon the Okinawa cases revealed interesting data. Serum neutralizing antibodies were questionably or certainly positive on the fourth or fifth day of the disease. Low titers of complement-fixing antibodies were found as early as two to eight days after the onset. However, these complement-fixing antibodies may not appear until the fifth week. No discrepancies were noted between the results of the neutralization and complement-fixing tests. For several technical reasons, Sabin feels that the complement-fixing test is more practical. It is interesting that many cases, initially suspected of being mild or abortive cases, in his Okinawa series gave no serologic evidence of Japanese B encephalitis.

*Pathology.*—Gross pathology reveals a diffuse congestion of the brain, sometimes with softening of the cervical cord.<sup>10</sup> There is diffuse involvement of the leptomeninges and the gray matter. Perivenous lymphocytes are predominant in the arachnoid meshes. The meningeal lesions are widespread over the surface of the brain, being minimal over the midbrain, pons, medulla, cerebellum, and spinal cord. Many nodules composed of mononuclear cells, clustered about degenerating ganglion cells or blood vessels, occur in the gray matter. The cortex, thalamus, substantia nigra, nuclei of the fourth ventricle, cerebellar cortex, and the anterior horns of the spinal cord are involved. Adrenal "tubular degeneration" may occur.

#### CASE REPORTS

**CASE 1.**—Patient T. H., a 23-year-old white man, entered the Navy Dispensary on Aug. 8, 1948, complaining of a headache beginning the previous day. This headache began suddenly, progressively became worse, and was accompanied by malaise and chills. He had completed the course of immunization against Japanese B encephalitis approximately two months before the present illness.

*Physical Examination and Laboratory Data:* T., 102.4° F.; P., 84; R., 22; B.P., 116/78 mm. Hg. The patient appeared flushed, dehydrated, and acutely ill. There was definite nuchal rigidity. The reflexes were physiologic, and Kernig's sign was negative. The spinal fluid showed 30 polymorphonuclear cells, and the Pandy reaction was negative. Blood leucocyte count on the second hospital day was 10,900 with 76 per cent polymorphonuclear cells. Admission urinalysis was negative. Initial chest films, as well as all subsequent chest films, were negative.

*Course:* Intravenous fluids, oral sulfadiazine, and intramuscular penicillin were instituted. On the second hospital day the fever rose to 104° F. There were 162 white cells in the spinal fluid, of which 127 were polymorphonuclear. During the next two days the temperature climbed to 104.6° F., and there was no essential change in the neurological picture. The patient was relatively alert. On the fourth hospital day the spinal fluid contained 133 polymorphonuclear and 98 mononuclear cells, and the Pandy reaction at this time was two plus. The patient became completely disoriented on the fifth hospital day. He did not respond to questioning, had to be restrained in bed, and was maniacal. Paraldehyde was necessary for sedation. There were definite neurological changes. The arms developed a lead-pipe type of rigidity, and there were marked gross tremors of the upper extremities and tongue with twitchings of the mouth. On the sixth hospital day, the temperature suddenly dropped and remained normal on the seventh day. The patient became more rational. A lumbar puncture at this time revealed approximately 400 cells in the spinal fluid, about three-fourths of them being polymorphonuclear, and a two plus Pandy reaction. Improvement after the seventh hospital day was rapid, and the temperature remained essentially

normal. During the subsequent thirteen days, the mental cloudiness, muscle twitching, and muscle tremors disappeared as well as all traces of nuchal rigidity. However, the patient's voice, which had become quite husky, remained so for two months. This has cleared completely at the time of the present writing, i.e., approximately five months from the onset of the illness. When the patient became ambulatory again, he complained bitterly of dizziness when walking, but this complaint rapidly disappeared.

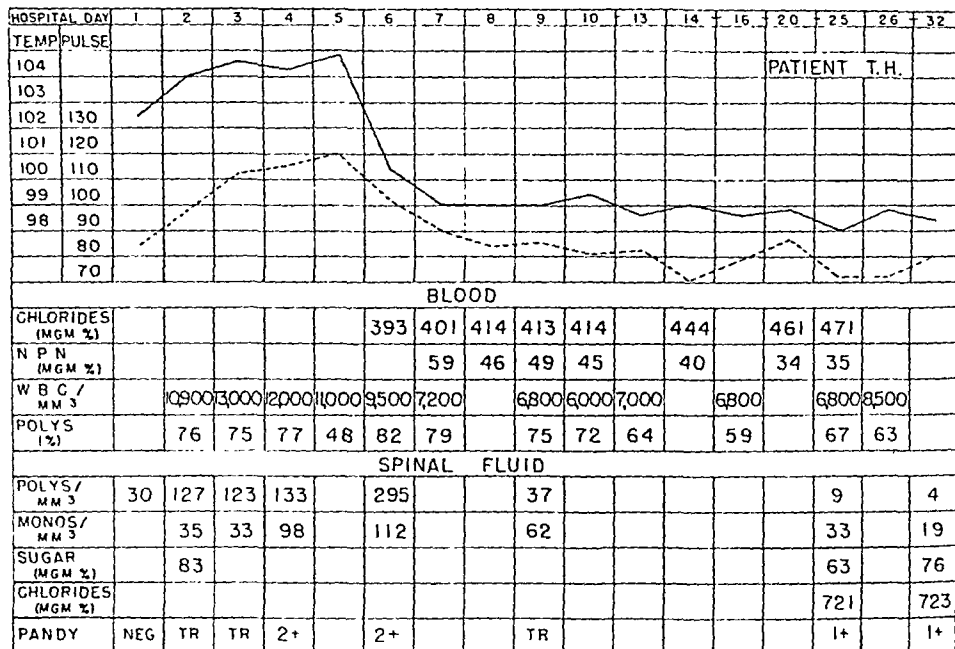


Fig. 1.

CASE 2.—Patient M. B., a 37-year-old white man, entered the Navy Dispensary on Aug. 28, 1948. The patient stated that the evening before he had experienced sudden severe headache and chills. He remembered starting to the Dispensary the next morning, but affirmed later that he did not have any recollection of his arrival nor of the immediately subsequent events. There had been no immunization against Japanese B encephalitis.

*Physical Examination and Laboratory Data:* T., 103.2° F.; P., 96; R., 22; B.P., 142/78 mm. Hg. Within a few moments after admission to the medical ward, the patient experienced a severe clonic convulsion with attendant cyanosis. The convulsion lasted approximately five minutes, and upon its cessation the patient was unruly and completely irrational, necessitating restraint by several men. The patient was disoriented and could make no intelligent reply to questioning. The administration of intravenous Sodium Amytal was necessary. There was flushing of the face and extreme dehydration. A foul discharge, from which pneumococci were subsequently cultured, drained from a right otitis externa. The mucous membranes of the throat were injected, and the tongue had been traumatized during the convulsion. A Grade II apical systolic murmur was present. There was marked nuchal rigidity. Kernig's sign was positive. All reflexes were hypoactive, the abdominal and ankle reflexes were absent, and the Babinski was positive bilaterally. The spinal fluid contained 425 cells, of which 321 were polymorphonuclear cells. The Pandy reaction was two plus. The initial blood leucocyte count was approximately 14,000 with 82 per cent neutrophils. The urine contained a trace of protein. The chest film was negative.

*Course:* Therapy, in the form of oral sulfadiazine, intramuscular penicillin, intravenous fluids, and intranasal oxygen, was instituted. On the second hospital day, the patient developed a lead-pipe rigidity of the arms and gross tremors of the lips and upper extremities. Similar but less marked rigidity of the lower extremities was also evident. This clinical picture persisted for six days. On the seventh day the temperature dropped to 99.8° F. The gross tremors of the lips, tongue, and arms were still present. The ankle jerks

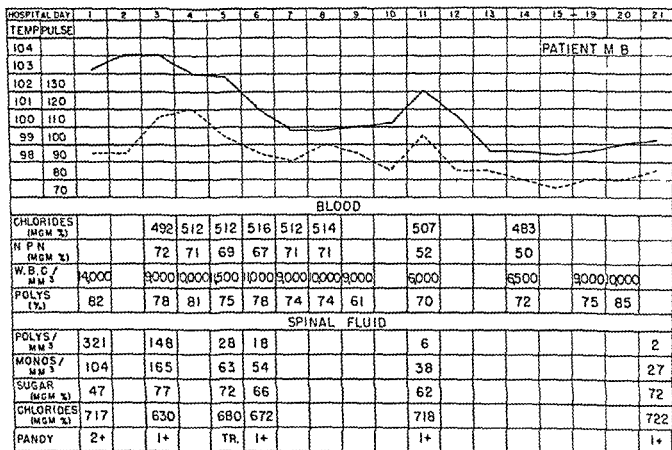


Fig. 2.

were absent, the Babinski was negative, and all other reflexes were hypoactive. Transient weakness of the external ocular rectus muscles appeared. It was necessary to discontinue oxygen for approximately thirty minutes because of a mechanical defect in the apparatus, and at the end of that time it was noted that the slightest stimulus could precipitate violent movements bordering on a convulsion. Following resumption of oxygen inhalation for a few minutes, the patient became much more responsive and much less hyperirritable. After the eighth hospital day he became much clearer mentally and rapidly regained his normal sensorium. Likewise, the muscle tremors cleared. Neurological abnormalities disappeared, and no physical sequelae of the encephalitis remained. His convalescence was complicated by a spondylitis.

**CASE 3.**—Patient W. P., a 24-year-old white man, entered the Navy Dispensary on Aug. 19, 1948, complaining of headache, fever, malaise, and nuchal rigidity of two days' duration. He had been immunized against Japanese B encephalitis in 1946 and had received a booster dose of 1 c.c. in 1947 and in June, 1948.

**Physical Examination and Laboratory Data:** T., 104° F.; P., 110; R., 24; B.P., 122/74 mm. Hg. The patient was acutely ill and markedly dehydrated. The skin was red and flushed. There was slight stiffness of the neck and transient weakness of the external rectus muscles of both eyes. All reflexes were normal, and the Babinski signs were normal. Initial spinal fluid studies revealed 143 white cells, 107 of them being polymorphonuclear, and a one plus Pandy reaction. The blood leucocyte count was 10,000 with 53 per cent polymorphonuclear cells. The urinalysis was negative. Initial and subsequent chest films were normal.



*Course:* Intravenous fluids, oral sulfadiazine, and intramuscular penicillin were administered. On the second hospital day the fever was  $104.2^{\circ}$  F., and there was no significant change in the clinical picture. On the third hospital day the patient complained of difficulty in breathing and became lethargic. Nuchal rigidity was more marked by this time, but there were no reflex changes. He became completely unresponsive the next day, at which time the temperature was  $104.5^{\circ}$  F. A lumbar puncture on this date revealed 256 polymorphonuclear cells and 241 mononuclear cells in the spinal fluid. The Pandy reaction at this time was two plus. There was no cyanosis nor evidence of cardiovascular impairment.

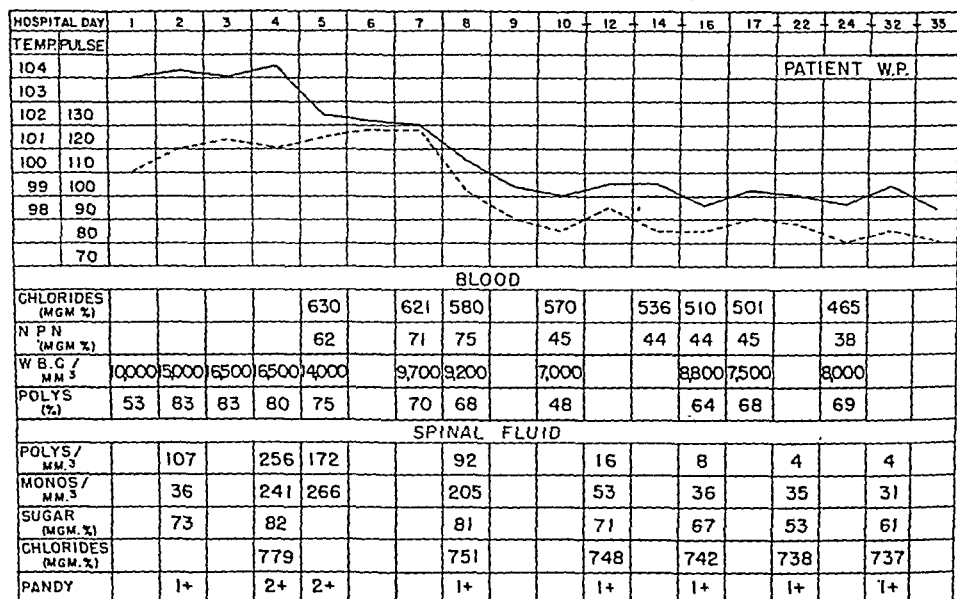


Fig. 3.

On the sixth hospital day the pulse rose to 128 and the respiratory rate accelerated to 30. There was marked tremor of the arms, lips, and tongue. Cheyne-Stokes respirations began and intranasal oxygen was administered. Later in the day the respiratory rate diminished to 22. At this time the respiratory movements were irregular but not Cheyne-Stokes in character. Knee jerks were hypoactive and the abdominal reflex was absent. Nuchal rigidity was definite, and the typical lead-pipe rigidity of the upper extremities was obvious. On the eighth hospital day, lumbar puncture revealed 92 polymorphonuclear and 205 mononuclear cells in the spinal fluid and a one plus Pandy reaction. On the evening of the same day, the patient experienced multiple convulsions, necessitating intravenous Sodium Amytal for their control. Cyanosis was demonstrable only immediately after the convulsions. Cheyne-Stokes respirations reappeared, associated with long periods of apnea. Pulmonary edema appeared suddenly, and the condition of the patient appeared grave. However, the pulmonary edema rapidly subsided after a hypodermic injection of morphine. Marked improvement occurred after this episode, and two days later the patient could respond to questioning. Twitching of the mouth, tremors of the upper extremities, and grimacing on minimal stimuli remained. The knee jerks and right ankle jerk could not be elicited. The temperature was now  $99^{\circ}$  F. By the thirteenth hospital day the patient was cheerful and alert. Both ankle jerks were now present, but the knee jerks were still absent. On this day the patient developed a seventh nerve palsy of the peripheral type on the right side of the face. This nerve weakness disappeared during the course of the next two weeks. Gross muscle tremors and twitches likewise receded. On the sixteenth hospital day the patient complained of

complete anesthesia of the dorsal and palmar surfaces of the index and middle fingers of the left hand. Neurological examination revealed no sensation in these areas. The sensory disturbance partially cleared in a few days, but marked weakness of the adductor of the left thumb and flexors of the left forefinger occurred. In a two-month follow-up, it was demonstrated that sensation over the fingers was normal and that the muscular weakness had improved on physiotherapy. Atrophy of the left thenar eminence was present.

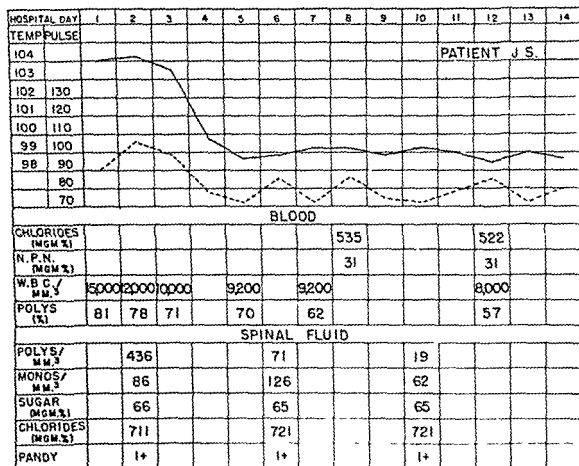


Fig. 4.

CASE 4.—Patient J. S., a 27-year-old white man, was admitted to the Navy Dispensary on Aug. 19, 1948, complaining of severe headache and vomiting. Four days previously he had noticed the sudden onset of headache, chills, and generalized malaise. During the twelve hours preceding admission he had experienced frequent emesis. His immunization against Japanese B encephalitis had been incomplete since he had received only two of the required three injections approximately two months before admission.

*Physical Examination and Laboratory Data:* T., 104° F.; P., 90; R., 22; B.P., 140/70 mm. Hg. The patient was quite dehydrated and flushed. He was, nevertheless, well oriented and alert. Moderate injection of the mucous membranes of the nose and throat was present. Nuchal rigidity was slight, though definite. The remainder of the neurological examination was negative. On the second hospital day there were 522 white cells in the spinal fluid, of which 436 were polymorphonuclear cells. The Pandy reaction was one plus. The initial blood leucocyte count was 15,000 with 81 per cent polymorphonuclears. The urinalysis was negative. Initial and subsequent chest films were negative.

*Course:* Therapy, consisting of intravenous fluids, oral sulfadiazine, and intramuscular penicillin, was started. The patient's course was uneventful, and on the second hospital day he appeared cheerful and alert. The temperature reached normal limits on the fifth hospital day, and nuchal rigidity cleared rapidly thereafter. During the mild course of the illness, no reflex changes nor clouding of the sensorium occurred. There were no tremors nor lead-pipe rigidity. On the fourteenth hospital day the patient was discharged to duty free of sequelae.

CASE 5.—Patient M. W., a 29-year-old white woman, entered the Navy Dispensary on Aug. 19, 1948, complaining of fever, malaise, and headache. These symptoms had begun suddenly three days before admission. The evening before admission she had become disoriented and irrational. There had been no immunization against Japanese B encephalitis.

*Physical Examination and Laboratory Data:* T., 105.5° F.; P., 120; R., 24; B.P., 110/80 mm. Hg. The patient was flushed and dehydrated. She was irrational and could not respond to questioning. A gross tremor of the upper extremities and twitching of the mouth

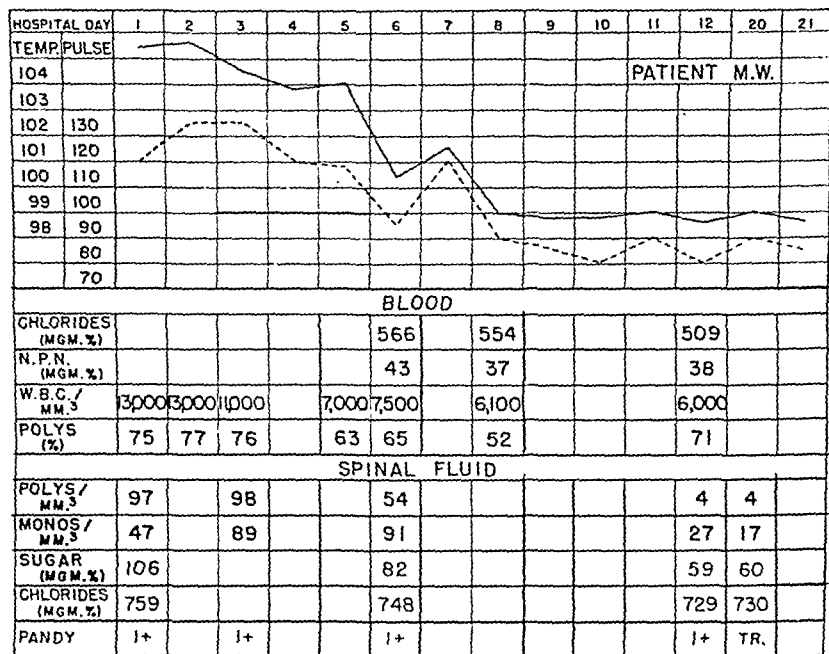


Fig. 5.

were present. Definite nuchal rigidity could be elicited. There was mild injection of the mucous membranes of the nose and throat. All reflexes were physiologic except for a questionably positive Babinski on the right. There were 97 polymorphonuclear cells and 47 mononuclear cells in the spinal fluid. The Pandy reaction was one plus. The blood leucocyte count was 13,000, 75 per cent of these cells being polymorphonuclear. The urinalysis was negative. Initial and subsequent chest films were negative.

*Course:* Intravenous fluids, oral sulfadiazine, and intramuscular penicillin were administered. During the following four days the patient remained delirious and continued to have the gross tremors described. Lead-pipe rigidity of the upper extremities also appeared. On the sixth hospital day the temperature decreased to 100.5° F. and on the seventh hospital day the patient became much clearer mentally. Thereafter improvement was rapid and all neurological abnormalities quickly disappeared. No sequelae remained.

#### DISCUSSION

The diagnosis in each of the five patients was proved by a rise in complement-fixing antibodies in the serum. The results of the complement fixation tests and the neutralization indices are shown in Table I.

Six other patients, having the same type of symptoms, spinal fluid pleocytosis, and clinical picture, are omitted from this discussion because positive serologic proof was not obtained. The question arises as to whether

TABLE I. COMPLEMENT FIXATIONS AND NEUTRALIZATION INDICES

PATIENT	HOSPITAL DAY	COMPLEMENT FIXATION*	NEUTRALIZATION INDEX
Case 1 T. H.	20	1:16	320
	26	1:64	1,000
	35	1:64	10,000
	†	1:64	2,000
	‡	1:32	10,000
Case 2 M. B.	6	Neg.	630
	17	1:8	20,000
	21	1:32	2,000
Case 3 W. P.	2	1:4	—
	9	1:4	10,000
	15	1:32	10,000
	26	1:32	200,000
	35	1:64	320,000
Case 4 J. S.	†	1:32	10,000
	2	1:64	6,300
	9	1:256	13,000
Case 5 M. W.	14	1:32	13,000
	2	Neg.	1,600
	9	Neg.	3,200
	15	Neg.	3,200
	21	1:8	10,000

\*A titer over 1:4 is considered significant.

†Follow-up twelve days after discharge

‡Follow-up five months after onset.

these were indeed cases of Japanese B encephalitis and whether it is possible that in some instances the threshold of immunologic response is not reached.

The therapeutic regimen adopted was necessarily varied to compensate for the individual needs of the patient. One of the characteristic features of the disease was the marked dehydration present initially. The nonprotein nitrogen determinations were elevated in two patients even after several days of rehydration and in a third before rehydration was accomplished. Probably the nonprotein nitrogen would have been elevated in the other two patients had this value been determined at the beginning of their hospitalizations.

Blood chloride values were generally within normal limits, and blood non-protein nitrogen determinations were consistent with the degree of dehydration evidenced by the patient. In no patient could it be demonstrated that renal disease was a factor in the elevation of the nonprotein nitrogen. A few fasting blood sugar determinations were normal. Several blood calcium determinations in two patients experiencing convulsions were normal.

Large quantities of saline and glucose were administered intravenously until adequate oral intake could be assured. As these individuals were quite young, large quantities could be infused with relative safety. In all cases, adequate urinary flow was achieved. In no instance did edema occur except in one patient who developed pulmonary edema following repeated convulsions. This edema quickly responded to morphine.

Intravenous barbiturate sedation was essential in two men to control multiple convulsions. Paraldehyde was requisite for one man who had become aggressive and maniacal.

Sulfadiazine and penicillin were instituted in all patients because, at that time, the exact nature of the disease had not been ascertained. It is

doubtful whether this therapy altered the course of the disease itself in any way. However, since among the Japanese bronchopneumonia was a common complication after the lysis of the encephalitic fever, chemotherapy may have had some value in the prevention of secondary infection.

One of the most valuable therapeutic adjuncts was oxygen. Many observers have expressed the opinion that the pathologic changes demonstrable at autopsy are to a large degree contingent upon anoxia. The salutary effect of oxygen was seen definitively in one patient. Patient M. B., Case 2, was receiving intranasal oxygen. After his initial convulsion had been controlled with Sodium Amytal and intranasal oxygen had been started, no further convulsions occurred even in the absence of further sedation. At one period in the illness, oxygen was discontinued for thirty minutes because of a mechanical defect in the apparatus. At the end of this period of time, the patient became much more restless and more delirious and upon slight stimulation developed convulsive movements which threatened to culminate in a generalized grand mal type of attack. Intranasal oxygen was then reinstituted, and after fifteen minutes the patient could respond to some questions intelligently, the hyperirritability of the central nervous system had subsided, and even strong stimuli failed to elicit convulsive movements. Oxygen was again discontinued temporarily, this time purposefully, and in half an hour the patient had reverted to his former state of lethargy and hyperirritability. Thereafter oxygen was administered continuously, and the clinical response was swift and gratifying. It is interesting to note that oxygen was almost specific when there were absolutely no signs of circulatory distress. The chest and heart were normal clinically and roentgenologically, and no clinical cyanosis nor respiratory distress was apparent.

A second patient, Patient W. P., Case 3, already receiving oxygen, experienced recurrent convulsions and developed pulmonary edema. Intravenous Sodium Amytal controlled the convulsions, and a hypodermic injection of morphine controlled the pulmonary edema. Thereafter, with intranasal oxygen, in the absence of further sedation, the patient remained calm and free of convulsions. The role of oxygen in controlling the convulsions in this case is less clear-cut than in that of Patient M. B., Case 2, but it was difficult to escape the clinical impression that oxygen was at least partially responsible in the suppression of convulsions. In retrospect, oxygen probably should have been administered to all patients.

Headache was a very distressing symptom. Frequently the simple procedure of withdrawing 10 c.c. of spinal fluid for study ameliorated the headache considerably. All of the patients had slight elevations of the spinal fluid pressure. Aspirin and codeine were valueless. Some clinicians of the occupation forces employed intramuscular caffeine and sodium benzoate with good results. We had no experience with this method of treatment.

Incontinence of urine and feces was a problem only so long as the patient was comatose.

The remainder of the therapy was purely symptomatic and similar to that employed in any acute infectious disease. Respiratory and intestinal isolation

techniques were instituted, but it is dubious whether the disease is contagious via the respiratory route. Those persons entrusted with the care of encephalitic patients in Japanese hospitals practice no precautions and seem none the worse for their multiple exposures. Throughout Japan there are extremely few instances of the disease occurring in more than one member of a family. The necessity of adequate mosquito screening seems obvious.

Many of the features of the disease can be discerned readily by a brief study of the charts. Necessarily, from such a small series, no sweeping conclusions can be drawn. These charts represent only the clinical picture in five patients seen at this Dispensary. Good correlation has been obtained, however, with much larger series occurring among Japanese nationals throughout the empire.

Initially, all patients were febrile, the height of the elevation ranging from 102° F. to 105° F. plus. Definite lysis of the fever occurred on the seventh day of the illness in two patients, on the eighth and ninth days in two others, and on the tenth day in one. Temperature lysis about the seventh day of the illness is characteristic of Japanese B encephalitis. It is interesting to attempt to correlate the degree of the febrile response with the prevailing disturbance of the sensorium. Four patients had markedly disturbed sensoria. The fifth, Case 4, Patient J. S., with an equally high temperature and with the most marked spinal fluid pleocytosis, was mentally alert and cheerful throughout his illness.

Relative bradycardia was present initially in only two patients. This quickly disappeared.

The blood leucocyte count varied from 10,000 to 15,000 on admission, and in the majority of these counts there was a decided "shift to the left." The count returned to normal in three to seven days.

Pleocytosis in the spinal fluid ranged initially from 30 to 552 cells. In four patients there was a preponderance of polymorphonuclear cells. In the fifth, no differential was done on admission. However, the next day the majority of cells in the spinal fluid were polymorphonuclear. The ratio of neutrophilic leucocytes to mononuclear cells was soon reversed, and subsequent spinal fluid examinations revealed a decided majority of mononuclear cells. The Pandy reactions were positive, semiquantitatively, from a trace to two plus. There were no strongly positive Pandy reactions in any stage of the disease. Spinal fluid sugars and chlorides were normal in all instances. Repeated smears of the centrifuged spinal fluid were negative for organisms. No bacterial growth was obtained on repeated cultures. Because of the difficulty in following these patients after their discharge from the Dispensary and in order to avoid repeated lumbar punctures, it was impossible to ascertain exactly the length of time requisite for all abnormalities to disappear from the spinal fluid. In the five patients, definite abnormalities were found on the tenth, twentieth, twenty-first, thirty-second, and thirty-second days respectively.

During the febrile episode there was transient, mild proteinuria, but this finding disappeared upon lysis of the fever.

The neurological picture during the course of the illness has been described in the case histories. Nuchal rigidity was present in all, although the rigidity was quite mild. In only one patient, Case 3, Patient W. P., did a cranial nerve palsy develop (seventh nerve), although a transient weakness of the external (lateral) ocular rectus muscles was present in Case 3, Patient W. P., and in Case 2, Patient M. B. All except one, Case 4, Patient J. S., experienced gross tremors of the upper extremities, lips, and tongue. It is noteworthy that the Japanese physicians consider a tongue tremor to be one of the first signs of recovery. This observation was not well correlated with the clinical course in this series. The ocular fundi were normal in all patients. Reflex changes were varied and transient. At various stages of the illness there were hyperactive, hypoactive, and absent knee jerks, ankle jerks, and abdominal jerks. Kernig's sign was positive in some but not all patients. The Babinski was transiently positive in some. There was rapid disappearance of neurological reflex abnormalities after lysis of the fever. All patients, except Case 4, Patient J. S., exhibited a lead-pipe type of rigidity of the upper extremities. The sensoria of all patients, except Case 4, Patient J. S., were markedly deviated from normal. These patients were restless and aggressive, with alternate periods of lethargy. This is in marked contrast to the extreme constant lethargy usually prevalent among native encephalitis victims.

One characteristic of the disease which deserves special emphasis is the rapidity of the patient's recovery. He may appear moribund, and the outlook may seem virtually hopeless. Even in such instances recovery may be swift.

There were no fatalities in this small series. The Japanese mortality rate of approximately 35.7 per cent is undoubtedly excessively high because of the poor methods of treatment in their hospitals. Even during the acute stage, while the patient is comatose, only 500 c.c. of fluid are administered each day by intramuscular drip, according to the practice in one of their large hospitals. This amount is not varied, regardless of the degree of dehydration, age, or size of the patient. "Acidosis" is "prevented" by the intravenous administration each day of 20 c.c. of a 50 per cent glucose solution. Oxygen, apparently, is infrequently, if ever, employed. A further disadvantage is the marked overcrowding of their hospitals with encephalitic patients, rendering very difficult the task of individualized attention by the staff of doctors.

Two patients in this series developed minor physical sequelae. In one, Case 1, Patient T. H., there was a definite change in the voice. The patient developed a very hoarse voice which improved after two months. No paralysis of the vocal cords has been demonstrated. The possibility of weakness of the superior laryngeal nerve was entertained. A second man, Case 3, Patient W. P., developed weakness of the adductor of the left thumb and flexors of the left forefinger. Initially, sensory perception was lost over the dorsal and palmar aspects of the second and third fingers of the left hand. The sensory abnormalities disappeared, leaving the residual muscle weakness outlined. Physiotherapy has greatly improved this condition.

The possible permanent mental changes resulting from infection with the encephalitic virus would comprise a most interesting and fruitful field for study. Limitations of time and lack of trained psychiatric consultants pre-

vented a detailed investigation of this important aspect of the disease. Lack of knowledge concerning the behavioristic pattern of the patient prior to his illness represents an intrinsic impediment in a study of this type. Without proper controls, only gross abnormalities can be attributed to the disease. None of these patients exhibited any marked impairment of the process of cerebration. Their intellectual ability was well within the normal range when tested by simple measures such as arithmetic problems, ability to exercise judgment in simple situations, and general knowledge.

Emotionally a similar pattern of dependence was evident in three of them. Even when the storm of the illness had passed and the patients were alert and recuperating remarkably, there was a strong reluctance on their part to attend in any way to their own needs. Only with difficulty, with the exception of one patient (Case 1, Patient T. H.), were they persuaded to abandon their recumbent positions. They were very loath to accept again the responsibility of bathing themselves. One patient, Case 2, Patient M. B., with a probable spondylitis, was extremely happy in his prolonged convalescence and almost refused to attempt anything for himself except the actual eating of his meals. However, the remainder of the patients did bow to the inevitable and, with one exception, Case 5, Patient M. W., were eager to return to their occupations. Case 5, Patient M. W., was anxious to return home provided she could convalesce for an indefinite period. Later it was ascertained that she promptly installed herself in bed at home and there remained for six weeks. The patient manifested extreme dependency, emotional lability, and was excessively demanding of those who attended her. According to one of her friends, she had definitely "changed" since her illness. Lapses in recent memory became obvious. Upon one occasion while entertaining guests, her husband left the room for a few moments. Almost coincident with his departure his wife confided in a friend her anxiety concerning him, as he had failed to return home that evening and had not called. Obviously, with no adequate preconfinement studies personality tendencies cannot indisputably be ascribed to the Japanese B virus.

One man, Case 1, Patient T. H., who least displayed this type of personality dependence, developed a curious propensity for inserting himself into odd and dangerous situations. He had a definite proclivity for wandering through the women's ward at night. He was content to confine his wanderings to the corridors and never did he enter any of the rooms. He had no explanation for his behavior. In one instance, he stopped the elevator between floors, climbed on top, and was reclining comfortably when discovered. Again, there was no adequate explanation. However, these bizarre quirks of behavior disappeared rapidly, and the patient was quite embarrassed when they were recalled to his attention. Interestingly enough, these peculiar phenomena occurred when the man seemed alert and well oriented. It seems possible that the encephalitic damage occasioned these transitory abnormalities.

All of the convalescent patients were unusually cheerful. Even when residues such as facial nerve palsy or tremors were evident, the patients ignored them magnanimously and refused to allow themselves to sink into the depths of depression. Only one patient, Case 5, Patient M. W., was emotionally labile,



but even she usually maintained a cheerful equilibrium. This emotional lability persisted in her for approximately six weeks after discharge.

The routine course of immunization comprised the administration of 1 c.c. of the chick embryo vaccine at weekly intervals for two doses followed by an additional 1 c.c. one month later. Case 4, Patient J. S., had only two injections; Case 2, Patient M. B., had none; Case 1, Patient T. H., had completed his course of immunization; Case 3, Patient W. P., had been immunized in 1946 and had received a booster dose of 1 c.c. in 1947 and 1948; Case 5, Patient M. W., had received no immunization.

#### SUMMARY

1. A series of five serologically proved cases of Japanese B encephalitis is presented. Six other patients with the same signs and symptoms were hospitalized during the epidemic but are not included in the series as no positive serologic proof could be obtained.

2. Only two of the five patients had completed their immunizations against Japanese B encephalitis.

3. The clinical features are outlined.

4. In addition to rehydration and sedation when necessary, oxygen seems to be a valuable therapeutic adjunct.

5. Only two minor physical sequelae occurred in five cases. There were no deaths.

6. The possibility of personality changes, resulting from the encephalitic virus, is discussed.

7. Improved treatment will probably reduce considerably the high mortality rate for Japanese B encephalitis prevalent among the Japanese.

The author wishes to express his appreciation to the Virus and Rickettsial Section, 406th Medical General Laboratory, Tokyo, Honshu, Japan, for the determination of complement fixations and neutralization indices.

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## PANCREATITIS IN INFECTIOUS MONONUCLEOSIS

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PANCREATITIS in association with mumps is often mentioned, though the literature on this point is not conclusive. Pancreatitis has been demonstrated definitely, however, in three patients with mumps, one at necropsy<sup>1</sup> and two at the time of surgical exploration.<sup>2, 3</sup>

There are few autopsy reports of cases of infectious mononucleosis. In eight case reports<sup>4, 5, 6, 7</sup> the pancreas was not mentioned. In three case reports<sup>8, 9, 10</sup> the pancreas was described as normal. There seems to be general agreement that infectious mononucleosis is a disease which may affect many organs.

Lucke and Mallory,<sup>11</sup> in their excellent study of the pathology of hepatitis, did not mention the pancreas. Capps,<sup>12</sup> however, mentions lesions occurring in the intestinal tract and in the pancreas. Comfort<sup>13</sup> noted in one case a persistent steatorrhea after infectious hepatitis and speculated on the association of pancreatitis with hepatitis.

In order to explore further the possibility that pancreatitis may be associated with other viral diseases, determinations of serum amylase and lipase were made at approximately weekly intervals on twenty consecutive patients with infectious mononucleosis and nine patients with viral hepatitis.

### MATERIALS AND METHODS

Serum amylase was determined by the Somogyi method.<sup>14</sup> Incubation times were determined to the nearest minute. The range of error in duplicate and triplicate determinations of the same specimen of normal serum was 0 to 36 per cent with a mean error of 6 per cent. Serum lipase was determined by the Cherry-Crandall<sup>15</sup> method as modified by Maelay.<sup>16</sup> Titrations were done with 0.1N NaOH to the nearest 0.1 cubic centimeter. The range of error on duplicate and triplicate determinations on the same specimen of normal serum was 0 to 50 per cent with a mean error of 14 per cent. The mean control amylase value based on thirty-six normal subjects was 146.4 units with a standard deviation of 55.7. Using three times the standard deviation as a criterion, the upper limit of normal for this group would be 313.5 Somogyi units. The mean control lipase value based on thirty-seven normal subjects was 0.27 c.c. with a standard deviation of 0.16. Using three times the standard deviation as a criterion, the upper limit of normal for this group would be 0.75 c.c. of 0.1N NaOH.

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## RESULTS

Two of the twenty patients with infectious mononucleosis presented abnormal serum enzyme values. In one patient values for both serum amylase and serum lipase were elevated, the highest serum amylase being 450 Somogyi units and the highest serum lipase being 1.7 c.c. of 0.1N NaOH. The second patient had abnormal values of serum lipase, the highest being 1.0 cubic centimeter. In this instance the values for serum amylase, although within the normal range, increased from 108 to 228 units. Liver function tests were done in nineteen of these patients and suggested an associated hepatitis in eighteen.

In nine patients with viral hepatitis, similar enzyme determinations at various stages of the disease gave normal results in all instances. Three were considered to be of the infectious type and six to be of the homologous serum variety.

Because of their particular interest, the two patients with infectious mononucleosis who, during the course of their illness, exhibited elevated serum enzyme values suggestive of an associated pancreatitis are presented in some detail. (Table I.)

CASE 1.—A 24-year-old, white, male school teacher, who had been in good health previously, was admitted to the hospital Jan. 3, 1948, complaining of headache and malaise of fourteen days' duration and of sore throat and fever up to 103° F. for one week. Penicillin and sulfadiazine had been administered by his family doctor without benefit. Physical examination revealed an acutely ill patient, the temperature being 103.4° F., and the presence of cervical, inguinal, and axillary adenopathy, marked tonsillitis, hepatomegaly, and splenomegaly. The hemoglobin concentration was 12.6 grams per 100 c.c. of blood and the leucocytes numbered 20,800 per cubic millimeter of blood. The percentages of the various types of leucocytes were as follows: neutrophils 30, lymphocytes 66 (many of them atypical), monocytes 2, basophils 1, and eosinophils 1. The Kahn test was 2 plus. Albuminuria was graded 2 plus and microscopic examination of the urine sediment revealed 3 to 6 leucocytes, occasional erythrocytes, hyaline casts, and granular casts per high-power field. Roentgenogram of the chest revealed moderate accentuation of the broncho-vascular markings bilaterally and a faint suggestion of pneumonitis in the left lower lung field. Bromsulfalein retention was 10.5 per cent at 45 minutes using a dose of 5 mg. per kilogram per body weight. The result of the cephalin flocculation test was 3 plus in 24 hours and 3 plus in 48 hours. The one-minute serum bilirubin was 0.2 mg. per cent and the total was 0.5 mg. per cent. Predominantly hemolytic staphylococci grew from a throat culture.

A clinical impression of infectious mononucleosis was corroborated by the relative and absolute lymphocytosis, the appearance of atypical lymphocytes in the peripheral blood smear, and a strongly positive heterophile antigen-antibody agglutination titer. The laboratory studies are presented in detail in Table I.

On the thirty-first day of illness the serum amylase was 450 units and the serum lipase was 1.7 cubic centimeters. In approximately twenty days these values had returned to normal. A glucose tolerance curve at the height of the elevation of serum amylase and lipase was normal.

Treatment consisted of bed rest, administration of 50,000 units of penicillin intramuscularly every three hours for the five days after admission, a high carbohydrate, low fat, and high protein diet with vitamin supplements and added brewers' yeast. The Kahn and Kolmer-Wassermann tests were negative after the patient had been two days in the hospital. The abnormal urinary findings disappeared after one week. A roentgenogram of the chest, taken ten days after admission, was normal. One week after penicillin therapy was discontinued a maculopapular rash appeared over the trunk. The liver was enlarged and tender for approximately one month after admission. There was never any abdominal

TABLE I. DETAILED CLINICAL AND LABORATORY DATA OF THE TWO PATIENTS WITH INFECTIOUS MONONUCLEOSIS WHO HAD ELEVATED VALUES OF SERUM AMYLASE AND LIPASE

DATE (1948)	DAY OF DISEASE	TEMPERATURE	RASH	TONSILLITIS	W.B.C. $\times$ 1,000	PER CENT POLYS	PER CENT LYMPHO- CYTES	HETERO- PHILE TITER	CEPHALIN FLOC- CULATION	SERUM BILI- RUBIN	BROM- SULF- ALEIN (%)	KAHN	SERUM AMYLASE	SERUM LIPASE
<i>Case 1</i>														
Jan. 3	15	103	-	3+	12.9	31	66	1:1,792				2+		
Jan. 6	18	102	-	2+	20.8	30	66		3+ 3+			Neg	450	1.7
Jan. 8	20	101	-	2+				1:1,792			10.5	Neg	400	1.3
Jan. 11	23	100	-	1+	16.5	10	86							
Jan. 15	27	99	+	-					3+ 3+	2-5				
Jan. 21	31	98.6	+	-	9.4	23	75	1:1,792						
Jan. 23	33	98.6	+	-	8.4	26	73							
Jan. 26	36	98.6	-	-										
Jan. 28	38	98.6	-	-										
Jan. 29	39	98.6	-	-										
Feb. 4	45	98.6	-	-										
Feb. 10	51	98.6	-	-										
Feb. 23		98.6	-	-	9.7	32	53							
<i>Case 2</i>														
Apr. 4	5	102	-	-	8.0	61	34							
Apr. 6	7	102	-	-	7.3	47	44	1:448						
Apr. 8	9	102	-	1+	11.7	29	65		3+ 3+	2-5		Neg	108	
Apr. 12	13	103	-	2+	16.8	30	60	1:896	3+ 4+	1-5				
Apr. 11	15	102	-	-					3+ 4+		11			
Apr. 19	20	100	-	-										
Apr. 21	22	98.6	-	-									158	1.0
Apr. 22	23	98.6	-	-										
Apr. 23	24	98.6	-	-									228	0.9
Apr. 25	27	98.6	-	-									200	0.6
Apr. 28	29	98.6	-	-									180	
Apr. 28	29	98.6	-	-									190	0.5
June 10		98.6	-	-					0 1+		1		120	

pain or diarrhea but occasionally the patient complained of backache. Appetite and strength gradually returned and the lymphadenopathy decreased. During the course of the illness the patient lost 23 pounds in weight.

CASE 2.—A 21-year-old, white student nurse who had previously been in good health was admitted to the hospital April 4, 1948, complaining of a slight cold and nasal congestion of five days' duration. Two days before admission she had noticed fever, fatigue, chilly sensations, and loss of appetite. Physical examination revealed an acutely ill patient with a temperature of 102° F. There was a slight mucoid nasal discharge. The throat was normal in appearance. The heart and lungs were within normal limits. A slightly enlarged, tender lymph node was found in the right anterior cervical region. The liver and spleen were not enlarged and there was no rash.

The hemoglobin concentration was 12.6 grams per 100 c.c. of blood and the leucocytes numbered 8,000 per cubic millimeter. The percentages of the various leucocytes were as follows: neutrophils 61, lymphocytes 34, monocytes 3, and eosinophils 2. The Kahn test for syphilis was negative. The urinalysis did not reveal abnormalities. Roentgenogram of the chest was normal. *Streptococcus viridans* and *Neisseria catarrhalis* grew from a throat culture. The heterophile antigen-antibody titer was 1:448. The cephalin flocculation test gave 3 plus results in 24 hours and 48 hours. The one-minute and total serum bilirubin concentrations were 0.2 and 0.5 mg. per cent respectively and the bromsulfalein retention with a dose of 5 mg. per kilogram of body weight was 11 per cent. Alkaline phosphatase was 10 King-Armstrong units. The prothrombin time was 44 per cent of normal. Total proteins were 6.2 grams per cent with 3.3 grams per cent of albumin and 2.9 grams per cent of globulin. The blood cholesterol was 134 mg. per 100 c.c. of serum with 42 per cent cholesterol esters. Bleeding time, clotting time, and platelet counts were normal. Two routine blood cultures and one blood culture with increased carbon dioxide tension were sterile. The blood was type O and Rh negative.

On the ninth day of illness the serum amylase was 108 Somogyi units and on the twentieth day, 158 units. On the twenty-second day the serum lipase was 1.0 c.c. of 0.1N NaOH and on the twenty-third day was 0.9 cubic centimeter. On the twenty-fourth day the serum amylase and lipase were 228 units and 0.6 c.c. respectively. Subsequent values for serum lipase rapidly returned to normal and the serum amylase decreased to 120 units. At this time the fasting blood sugar was 96 mg. per cent and the urinalysis for sugar was negative.

Subsequent development of an absolute lymphocytosis, the appearance of atypical lymphocytes in the stained peripheral blood smear, and a strongly positive heterophile antigen-antibody titer established the diagnosis of infectious mononucleosis. See Table I for complete details of the laboratory studies.

On the tenth day of disease numerous petechiae were noted on the hard palate which persisted approximately four days. About the same time the normal pharynx became injected in appearance, the tonsils were covered by a grayish-white membrane, and at this time the predominant organism on throat culture medium was nonhemolytic streptococcus. The sore throat accompanied by headache and pain on moving the eyes had nearly disappeared when, on the fifteenth day of illness, the patient became markedly anorexic and nauseated and vomited repeatedly. Treatment at this time entailed administration intravenously of solutions containing glucose, Parenamine, B complex vitamins, ascorbic acid, and Synkayvite. The liver became tender to percussion but still was not enlarged. The patient became afebrile on the twenty-second day of illness and by the twenty-ninth day she was sufficiently improved to be discharged to her home for gradual resumption of activities. She had lost at least 8 pounds in weight.

#### SUMMARY AND CONCLUSIONS

1. Twenty consecutive cases of infectious mononucleosis were studied at weekly intervals and in two instances a definite elevation of the values of serum amylase and lipase was demonstrated. This finding is suggestive of an as-

sociated pancreatic disturbance which to our knowledge has not been described previously in patients with infectious mononucleosis. This was possibly a pancreatitis, but lymph node obstruction of the pancreatic ducts must be considered.

2. In nine cases of viral hepatitis the serum amylase and lipase determinations were made at varying intervals and no elevated values were found. No conclusions can be drawn from this small series except that there was no evidence for pancreatitis at the time the determinations were made. This series should be extended and the possibility of pancreatitis occurring in other viral diseases should be explored.

3. It is quite possible that many asymptomatic, acute, subacute, and chronic inflammatory changes of the pancreas exist in association with viral diseases which at the present time are not appreciated. It is conceivable that such infection may play a role in the etiology of chronic relapsing pancreatitis, the pathogenesis of which remains obscure.

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# A CASE OF CONGENITAL IDIOPATHIC METHEMOGLOBINEMIA

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THE differential diagnosis of cyanosis in the adult does not often present a difficult problem except in the instance of chronic, persistent cyanosis which does not vary. This is more interesting if the patient is middle aged or older, and if the cyanosis has been present during the greater part of his life. Proper consideration must be given to cyanosis produced by altered hemoglobin compounds within the circulating blood. The nature of the compound and its concentration in the blood are important in the diagnosis, treatment, and prognosis of these disorders. The three pigments (always intracorporeal) which most frequently produce cyanosis are: reduced hemoglobin (as in cardiopulmonary disease), methemoglobin, and sulfhemoglobin. When these are present in the blood in concentrations of 5, 1.5, and 0.5 Gm. or less per 100 ml. of blood, respectively, cyanosis may be clinically evident.<sup>1</sup> Minute amounts of methemoglobin (0.03 to 0.13 Gm. per 100 ml.) have been reported in the blood of normal persons.<sup>2</sup> This may be a normal stage in the degradation of the pigment. The compounds have several definite chemical and physical properties by which they may be identified.<sup>3</sup>

Clinically, methemoglobinemia may be primary or secondary. The secondary type is more common and is most often due to drug toxicity. Anemia and/or granulocytopenia are usually accompanying findings in chronic cases, and the systemic effects are striking. Of much less frequent occurrence is the primary type. There is no anemia; indeed, a polycythemia is often present apparently as a compensatory measure. Systemic symptoms are exceptionally mild, and a familial incidence has been noted in many of the cases reported. Since the condition is rare, the following case is believed to be of interest.

## CASE REPORT AND EXPERIMENTAL STUDY

A 61-year-old white man was admitted to the Marine Hospital with complaints of failing vision in both eyes. On physical examination, bilateral cataracts were discovered. It was also noted that the patient had a marked cyanosis of the mucous membranes, lips, and fingers, and a slate gray color of the skin all over the body. There was no clubbing of the digits, and the conjunctival and retinal blood vessels were engorged. The patient stated that his blue-gray skin coloring had been present "since birth," and that people had frequently remarked about it when he was a child. No other familial instance could be elicited. There was no history of prolonged drug ingestion, and system review failed to disclose any abnormality of gastrointestinal function. The patient had no complaints other than his visual disturbance, and cardiopulmonary disease was ruled out by physical examination, x-ray and fluoroscopic studies, and normal electrocardiographic tracings.

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The patient was seen in consultation by Dr. A. J. Beams,\* who suggested a diagnosis of idiopathic methemoglobinemia. A sample of blood was examined and found to contain: total hemoglobin, 21.9 Gm. per 100 ml; oxyhemoglobin, 14.3 Gm.; and the difference, 7.6 Gm., was nonfunctional hemoglobin which showed a spectroscopic absorption band similar to that of a known methemoglobin solution. Laboratory findings at this time revealed nothing of significance except an icterus index of 17.6 units; red blood cells, 6,350,000 per cubic millimeter; and a hematocrit of 68 per cent packed red cells. The red cell indices revealed a mean corpuscular volume of 106 c.micra and mean corpuscular hemoglobin concentration of 29.2 per cent. The white blood cells were normal.

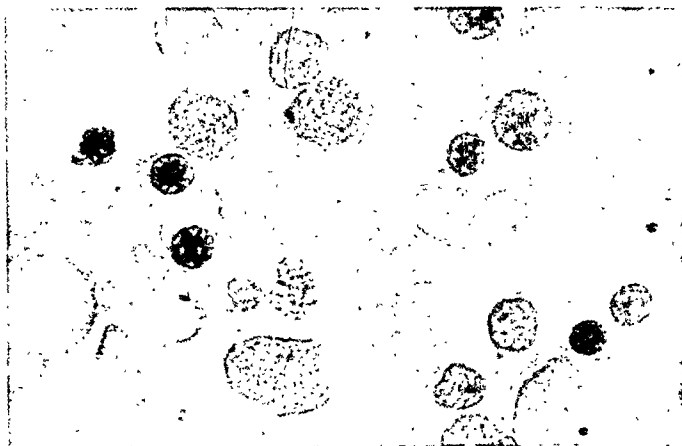


Fig. 1.—Sternal bone marrow ( $\times 950$ ) showing normoblastic hyperplasia.

The patient was operated on for a cataract of the left eye and then discharged from the hospital. Four months later he was asked to return to the hospital and was admitted to the medical service for further study of his methemoglobinemia. On this second admission he had no complaints and was not dyspneic. The physical examination was essentially the same as on the first admission except for a partial left iridectomy and absence of that lens. The blood pressure was 150/94, temperature 96.2° F., pulse 50, and respirations 24. The red blood cell count was 5,930,000 per cubic millimeter, with a hemoglobin of 18.9 grams. The hematocrit was 61 per cent. When venous blood was drawn it had a dark, chocolate-brown color. Exposure of the blood to air over a period of twenty-four hours produced no noticeable change in color, and long periods of oxygen inhalation did not affect the patient's cyanosis. The reticulocytes numbered 0.7 per cent, and the icterus index was 13.0 units. The total hemoglobin measured 19.9 Gm., oxyhemoglobin, 12.5 Gm., and methemoglobin, 7.4 Gm. per 100 ml. of blood prior to the beginning of therapy. Aspiration of sternal bone marrow yielded a moderately thick marrow which was also chocolate-brown in color. The centrifuged marrow showed fat, 5 per cent; plasma, 32 per cent; buffy (myeloid: erythroid) layer, 9 per cent; and red cells, 53 per cent. Marrow smears showed an erythroid hyperplasia at the normoblast level. (Fig. 1.)

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X—X TOTAL HEMOGLOBIN

# METHEMOGLOBIN

(NUMBERS IN PARENTHESES INDICATE % OF METHb IN TERMS OF TOTAL Hb)

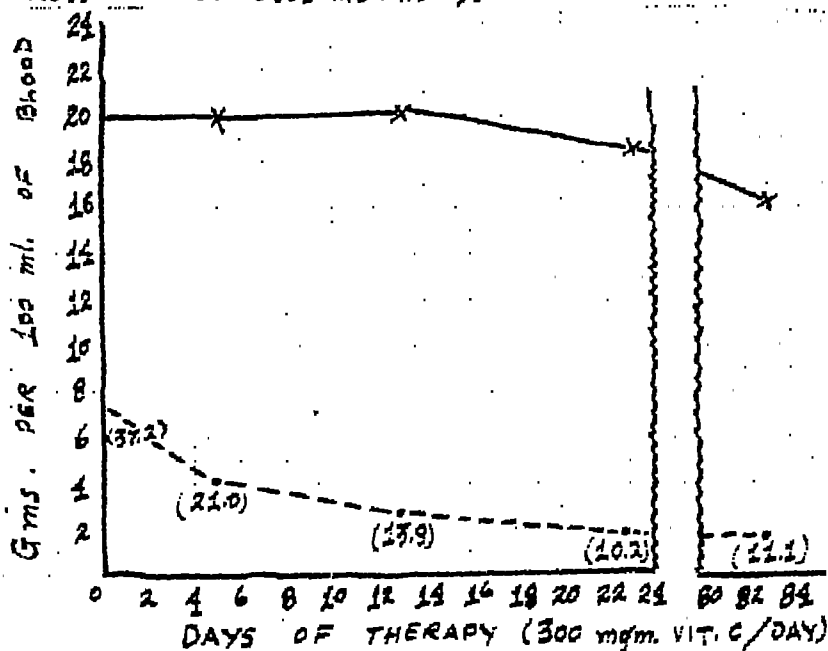


Fig. 2.—The effect of ascorbic acid on methemoglobin.

The patient was then started on oral treatment with ascorbic acid in doses of 300 mg. each day. Clinical improvement was slow at the start but easily noticeable by the third day of therapy. On the fifth day the patient noted the change in his color, and the venous blood appeared more red. At the end of ten days, the labial and gingival cyanosis was almost gone. By the end of the second week, the total blood methemoglobin had been reduced to almost one-half (Fig. 2). The laboratory findings during this therapy are summarized in Table I; the changes in methemoglobin are shown in Fig. 2.

This therapy was continued for one month, during which time the patient's skin color became more pink. The venous blood appeared almost normal in color, and the methemoglobin level dropped to 1.9 Gm. per 100 milliliters. By the end of this time, the patient

TABLE I. LABORATORY DATA DURING PERIOD OF THERAPY

[illegible]

wished to be discharged from the hospital. He was placed on outpatient status and given a supply of ascorbic acid, but he failed to return for follow-up. Two months later he returned for more ascorbic acid, and further data were then obtained. His skin color was definitely a normal pink hue, and the venous blood had a normal dark red color. The laboratory findings at this time are listed as "83rd day" in Table I and Fig. 2. The bone marrow showed no significant change.

#### DISCUSSION

This case was of interest for several reasons: the chronicity of the illness, the high initial methemoglobin level, and the response during the period of therapy. The patient was able to carry on strenuous work over a period of years, as a merchant seaman, without dyspnea, fatigue, or other constitutional symptoms. Finch,<sup>1</sup> who reviewed this subject exhaustively in 1948, stated that he saw a patient with a methemoglobin level of 40 per cent who was able to carry on strenuous work; our patient had 37.2 per cent. A more remarkable point of interest arose during the course of therapy. Although the usual response with treatment is a reduction in methemoglobin and total hemoglobin, our patient exhibited a different course. As the methemoglobin decreased, the total hemoglobin remained stationary and the oxyhemoglobin level rose. The red cell count and hematocrit also remained fairly stationary. This was entirely contrary to our expectations, since we anticipated hemolysis of the excess erythrocytes as the methemoglobin was reduced. Undoubtedly the chronic anoxemia present for so many years proved a potent stimulus to the erythrogenic elements of the bone marrow. Had we been able to follow the patient for a longer period, it would have been interesting to note how long a time elapsed before the number of circulating red cells became reduced in number.

At least sixteen proved cases of congenital idiopathic methemoglobinemia had been reported in the literature up to the time of Finch's review (1948). In eight, there was a familial incidence. In 1947, Coudounis and associates<sup>2</sup> reported fourteen cases, all occurring in a Greek family within four consecutive generations; four pairs of siblings were affected by the syndrome. The name of the family was Vophtochilari, which the authors translated as "people with discolored lips." Sievers and Ryon<sup>3</sup> stated that they were able to tabulate eighteen reported cases up to 1945, and they added one of their own. Gibson and Harrison (1947)<sup>4</sup> reported five cases in one family. Bensley, Rhea, and Mills (1938)<sup>7</sup> reported two cases occurring in a brother and sister and stated that these were the first in which the diagnosis was established definitely. They added that the cyanosis was constant and was not affected by any form of therapy; vitamin C was not used.

Other cases have been reported in the literature from time to time. In some, history of drug intoxication could not be ruled out. Others were given the name of "enterogenous cyanosis," supposedly associated with constipation and production of large amounts of nitrites by the bacteria of the intestinal tract.

To Lian<sup>8</sup> is ascribed the first use of ascorbic acid in the treatment of methemoglobinemia. It was believed to be superior to methylene blue. Ascorbic

acid has been used in many of the cases reported since then, almost all with excellent results.<sup>10</sup> The oral route is as effective as the parenteral, and Graybiel and co-workers<sup>9</sup> have found that concomitant administration of sodium bicarbonate decreases the urinary excretion of vitamin C and the rate at which it disappears from the blood. King, White, and Gilchrist<sup>10</sup> found that regardless of the amount of ascorbic acid given to their patient, the methemoglobin level could not be reduced below 7 per cent of the total blood pigment. Gibson and Harrison<sup>6</sup> also found that they were not able to reduce the total methemoglobin below 1.0 Gm. per 100 ml. of blood by the use of vitamin C. Our findings, over a short period of time, are similar to these.

Normally, the mammalian anuclear erythrocyte reduces methemoglobin by an enzymatic process in which glucose and lactate are the principal substrates. Clinical methemoglobinemia may then be produced by absence or failure of this reconversion mechanism (primary) or by the action of oxidants which produce methemoglobin more rapidly than the cell is able to reduce it (secondary). Further, the oxygen dissociation curve is normal in primary methemoglobinemia, whereas that induced by drugs shows a shift to the left.<sup>1</sup>

Gibson proposes that the erythrocytes of primary methemoglobinemic patients are deficient in coenzyme factor I, which functions with triose phosphoric and lactic enzymes of the red blood cell to facilitate reduction of methemoglobin to hemoglobin. Methylene blue is therefore thought to catalyze reduction of methemoglobin by enzymes otherwise unable to bring about this reaction. Ascorbic acid, by contrast, reduces methemoglobin directly, but is never complete.<sup>6</sup>

These differential diagnostic features are important in providing the proper therapy for methemoglobinemia.

#### SUMMARY

A case of congenital idiopathic methemoglobinemia in a 61-year-old man is presented. The patient was treated with daily oral doses of 300 mg. of ascorbic acid and showed an excellent, though somewhat unusual, response to therapy. The case is of interest because of the age of the patient, the chronicity of the disease, and the trend during therapy. Some of the pertinent literature is reviewed briefly.

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## LABORATORY METHODS

### A SIMPLIFIED TURBIDIMETRIC METHOD OF AUREOMYCIN ASSAY FOR CAPILLARY BLOOD AND OTHER BODY FLUIDS

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FROM studies on various methods of bio-assay for aureomycin, a turbidimetric method has evolved which is sufficiently accurate and sensitive in the presence of body fluids for investigative purposes and is sufficiently simple for routine use. It also meets a prime requirement for pediatric use in that an amount of blood adequate for serum assay can be obtained from a single finger or heel puncture. Osgood and Graham<sup>1</sup> developed a similar turbidimetric method of assay for penicillin, streptomycin, and trivalent organic arsenicals. Meads and co-workers<sup>2</sup> adapted it to the assay of aureomycin. We found that for aureomycin the accuracy of this method in the presence of body fluids is increased appreciably by substituting a standard curve containing four points for the single standard used by the original workers and that this change also simplifies the method by eliminating most of the mathematical details involved in computing the results.<sup>3</sup> Several other minor modifications were also helpful. It therefore seems worth while to publish the details of this method as it is being used in our laboratory.

#### DETAILS OF TECHNIQUE

**Materials.**—A Klett-Summerson colorimeter with a No. 660 filter; sterile matched colorimeter tubes with rubber stoppers, 0.85 per cent saline, 1 and 5 c.c. pipettes; a 37° C. water bath, an ice water bath in the refrigerator, and standardized, uncontaminated test organism (Oregon J strain of *Staphylococcus aureus*—American Type culture collection No. 9801) are used.

The organism is standardized as follows: 5 ml. amounts of a fresh one- to three-hour broth culture\* are added to 100 ml. amounts of sterile Bacto-Tryptose Phosphate Broth (Difco), which is then incubated in the 37° C. water bath until the optical density of the culture as determined by colorimeter readings at fifteen-minute intervals is approximately fifteen points (twelve to eighteen points) greater than that of a broth blank. This usually takes two and one-half to three hours. At this point, growth is stopped by transferring the culture to the ice water bath. This, the standardized culture, is kept in the ice water bath until used and is made up fresh every four days.

From The Children's Hospital of Philadelphia (Department of Pediatrics, School of Medicine, University of Pennsylvania).

The aureomycin used in this study was supplied by Lederle Laboratories Division of the American Cyanamid Company.

These studies were conducted under contract with the Department of the Army, Chemical Corps, Camp Detrick, Maryland.

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\*This is prepared by inoculating Bacto-Tryptose Phosphate Broth from the stock culture. The stock culture is kept on a nutrient agar slant in the refrigerator. A fresh slant is prepared every two weeks. Each new slant is checked for contamination before use.

A series of previously matched, cotton-plugged, sterile colorimeter tubes is arranged for assay as shown in Table I. As soon as all dilutions are made, sterile rubber stoppers are inserted into the tubes and the solutions agitated by inversion of the tubes. The colorimeter is set at zero with the blank tube, and the turbidity of each remaining tube is read rapidly in a predetermined order. Immediately after reading, each tube is placed in the 37° C. water bath. Four hours later each tube is individually removed from the water bath, wiped, agitated, and reread in the same order.

TABLE I. MATERIALS ADDED TO TUBES FOR TURBIDIMETRIC ASSAY OF AUREOMYCIN IN BODY FLUIDS

TUBE	APPROPRIATE BODY FLUID* (ML.)	SALINE (ML.)	MICROGRAMS OF AUREOMYCIN PER ML. OF SALINE†		STANDARDIZED TEST CULTURE‡ (ML.)
			FOR URINE ASSAY	FOR SERUM AND SPINAL FLUID ASSAY	
Blank	0	5.0	0	0	0
Control	0.5	0.5	0	0	4.0
Standard 1	0.5	0.5	2.0	1.0	4.0
2	0.5	0.5	1.0	0.5	4.0
3	0.5	0.5	0.5	0.25	4.0
4	0.5	0.5	0.25	0.125	4.0
Unknown 1	0.5	0.5	0	0	4.0
2	0.5	0.5	0	0	4.0
Etc.	0.5	0.5	0	0	4.0

\*Contains no added aureomycin.

†Aureomycin dilutions freshly made from a 5,000 µg per milliliter of aureomycin solution, which is made up fresh every two weeks from lyophilized aureomycin and kept at 4° C.

‡Added last while still at 4° C.

Computation of the results hinges on the principle demonstrated by Osgood† that, in the case of a sensitive organism, when sufficient bacterial growth has occurred to provide for differences that can be accurately measured, the concentration of the appropriate antibiotic is proportional to the square of the difference between the turbidity of a control tube and the turbidity of a tube containing the antibiotic. The details are described as follows and are illustrated in Table II. The increase in turbidity between the four-hour reading and the zero-hour reading is determined in each of the four standard tubes (containing known amounts of aureomycin), each of the unknown tubes (containing the body fluid to be assayed), and the control tube (containing no aureomycin). The increase in turbidity (which is the measurement of bacterial growth during the four hours of incubation) of each standard tube and each unknown tube is then subtracted from the increase in turbidity of the control tube. The difference in turbidity increase between the control

TABLE II. COMPUTATION OF RESULTS FOR A SERUM STANDARD CURVE

TUBE	AUREOMYCIN (γ PER ML.)*	TURBIDITY IN KLETT UNITS		TURBIDITY INCREASE (4 HR.-0 HR. READING)	DIFFERENCE IN TURBIDITY INCREASE BE- TWEEN CON- TROL AND STANDARD	DIFFERENCE IN TURBIDITY INCREASE BE- TWEEN CON- TROL AND STANDARD, SQUARED
		0 HR.†	4 HR.			
Control	0	46	133	87		
Standard 1	1.0	47	70	23	64	4,104
2	0.5	47	85	38	49	2,401
3	0.25	44	102	58	29	841
4	0.125	46	121	75	12	144

\*Concentration in saline added to tubes. Final concentration is one-tenth of this.

†This reading corrects for slight differences in optical density of the individual tubes and individual specimens.

tube and each of the standard and unknown tubes is squared and is, by definition, proportional to the amount of antibiotic present. The squared values for the standards are plotted along the abscissa of semilog paper against the known concentrations of aureomycin per milliliter of saline (see Fig. 1 and Table I). A standard curve is drawn through the four points obtained. The concentration of aureomycin in the unknowns is read off this curve using the squared values for the unknowns obtained as described.

#### SERUM STANDARD CURVE

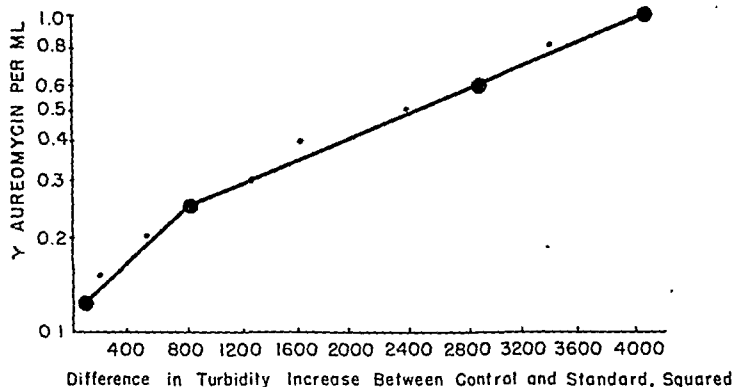


Fig. 1.—The four standard points are plotted in large dots, while twelve simultaneously determined intermediate values are plotted in small dots. The concentrations of aureomycin plotted on the ordinate of this graph are ten times the final concentration of aureomycin in the standard tubes and therefore correspond to the concentration of aureomycin in an unknown solution before it is diluted by addition of saline and standardized test culture (see Table I).

Blood is collected from a finger or heel puncture and the serum is separated for assay as previously described by one of us (A.D.H.).<sup>4</sup> The collecting tube is made either from a three-inch length of glass tubing, with one end sealed, or from a 7.5 by 95 mm. shell vial. The open end is drawn out to a capillary tip and sealed. The wide portion of the tube is held over the flame and an air vent is quickly produced in the softened glass by the expanding air within the sealed tube. For the collection of blood, the capillary tip is broken off near its base. The finger or heel is punctured so that a free flow of blood is obtained. The tube is held in as nearly vertical a position as possible and the blood readily flows into the tube and down to the base. The tubes are stood upright until the blood is clotted; then the tube is filed off just above the surface of the blood. The clot is broken up with an applicator stick and the tube is centrifuged at 1,500 to 2,000 revolutions per minute for fifteen minutes. The supernatant serum is then pipetted off with a capillary pipette for assay. After a little practice with this technique, one can consistently collect 1.5 ml. or more of capillary blood from a single skin puncture.

Unknown serum and spinal fluid specimens are run in 1:1 and 1:8 dilutions,\* thus bringing a maximum of 8  $\mu$ g per milliliter of aureomycin within the detectable range of the method. Similarly, unknown urine specimens are run in 1:20 and 1:160 dilutions to detect a range of 5 to 320  $\mu$ g per milliliter of aureomycin. These predilutions are made in the type of body fluid being assayed. Serum† for them is obtained from the blood bank,

\*For maximum accuracy, a separate pipette is used for each step in the dilution of serum specimens.

†Twenty individual specimens of human serum were tested simultaneously under the conditions of this method and no significant difference was found in the growth of the test organism among these sera. Serum, however, does stimulate the growth of the test organism markedly when compared with the assay broth. Keeping the serum concentration constant in all the tubes of a serum assay controls this factor. However, the concentration of aureomycin in an unknown specimen is calculated from the tube with the greatest predilution when there is a choice, since most of the body fluid in prediluted specimens comes from the same source as the body fluid used in the standard and control tubes.

spinal fluid from air encephalographic studies, and urine from laboratory personnel. All body fluid for dilution of unknown specimens is kept frozen until used. Correction for such dilutions is made in calculating the results.

Aureomycin in a concentration of 1  $\mu\text{g}$  per milliliter in serum and 40  $\mu\text{g}$  per milliliter in acid urine shows no appreciable loss of activity when kept at  $-20^{\circ}\text{C}$ . for one week. However, aureomycin in spinal fluid in a concentration of 0.25  $\mu\text{g}$  per milliliter shows about a 50 per cent loss of activity if kept at  $-20^{\circ}\text{C}$ . overnight. Therefore, unknown specimens of serum and acid urine are kept at  $-20^{\circ}\text{C}$ . until a convenient time for assay, but spinal fluid unknown specimens are assayed on the day collected. The pH of all urine used is adjusted to 7.2 just before assay.

Replicate determinations of either controls, standards, or unknowns do not appreciably enhance the accuracy of the method. A separate standard curve is required for each type of body fluid on each day of assay.

### RESULTS

The results of one hundred and four consecutive determinations of known concentrations of aureomycin intermediately spaced between the values used to make up the standard curve are tabulated in Tables III, IV, and V. One experiment using serum is illustrated in Fig. 1 in which it will be noticed that the standard curve approaches a straight line on semilog paper. A similar straight line is obtained with the other two body fluids tested. The percentage error of each determination has been computed. For urine, the range of error is 0 to 42 per cent, with a mean of 12.4 per cent; for spinal fluid, the range is 0 to 35 per cent, with a mean of 12.3 per cent; for serum, the range

TABLE III. RECOVERY OF KNOWN CONCENTRATIONS OF AUREOMYCIN IN URINE FROM 36 CONSECUTIVE DETERMINATIONS

KNOWN $\gamma$ AUREOMYCIN PER ML.	1.60	1.50	0.80	0.60	0.40	0.30
Recovered $\gamma$ aureomycin per ml.						
Determination 1	1.48	1.20	0.84	0.56	0.36	0.26
2	1.48	1.33	0.84	0.60	0.39	0.27
3	1.50	1.33	0.86	0.62	0.50	0.33
4	1.55	1.40	0.90	0.62	0.50	0.36
5	1.55	1.40	1.00	0.73	0.50	0.38
6	1.75	1.55	1.00	0.73	0.50	0.48

TABLE IV. RECOVERY OF KNOWN CONCENTRATIONS OF AUREOMYCIN IN SERUM FROM 36 CONSECUTIVE DETERMINATIONS

KNOWN $\gamma$ AUREOMYCIN PER ML.	0.80	0.60	0.40	0.30	0.20	0.15
Recovered $\gamma$ aureomycin per ml.						
Determination 1	0.60	0.56	0.35	0.26	0.14	0.13
2	0.74	0.60	0.36	0.28	0.16	0.13
3	0.77	0.60	0.39	0.29	0.18	0.14
4	0.78	0.62	0.41	0.31	0.19	0.14
5	0.78	0.62	0.42	0.31	0.20	0.14
6	0.92	0.71	0.45	0.35	0.20	0.15



TABLE V. RECOVERY OF KNOWN CONCENTRATIONS OF AUREOMYCIN IN SPINAL FLUID FROM 32 CONSECUTIVE DETERMINATIONS

KNOWN $\gamma$ AUREOMYCIN PER ML.	0.87	0.80	0.75	0.62	0.60	0.40	0.37	0.30	0.20	0.15
Recovered $\gamma$ aureomycin per ml.										
Determina- tion										
1	0.67	0.73	0.47	0.53	0.57	0.33	0.29	0.25	0.17	0.13
2	1.00	0.78	0.47	0.53	0.62	0.33	0.29	0.26	0.19	0.13
3	-	0.78	-	-	0.62	0.40	-	0.30	0.20	0.14
4	-	0.78	-	-	0.68	0.44	-	0.32	0.20	0.14

is 0 to 35 per cent, with a mean of 8.3 per cent. The accuracy of this method appears to be distinctly superior to that we achieved, in the presence of body fluids, with the original turbidimetric method of Meads and a serial dilution method of aureomycin assay.<sup>3</sup> Furthermore, it is possible to detect 0.1  $\mu\text{g}$  of aureomycin per milliliter or less in the presence of the three body fluids tested, so that its sensitivity compares favorably with the sensitivity of the other methods currently in use.<sup>3</sup> The method is not as accurate in concentrations below 0.125  $\mu\text{g}$  per milliliter as in higher concentrations, however.

#### CONCLUSION

A modification of Meads' turbidimetric method of aureomycin assay has been described. It appears to provide a simple and accurate means of assaying low concentrations of aureomycin in small volumes of various body fluids.

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## A MICROMETHOD FOR BLOOD PENICILLIN ASSAY

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IN MAKING frequent penicillin plasma determinations in children, it is helpful if a method can be used which requires such a small amount of blood for each assay that it can be obtained from a puncture wound of the finger as opposed to withdrawal by venipuncture. Fielding<sup>1</sup> described a micromethod requiring as little as 0.25 ml. of fluid for assay in which the test organism was the Oxford strain of *Staphylococcus aureus* grown in glucose serum medium containing Andrade's indicator. The principle of the test was the detection, by means of the color indicator, of the pH change induced in the medium by the breakdown of glucose during the growth of the test organism. When the growth of the organism was prevented by a given amount of penicillin, then no color change would occur. The minimum amount of penicillin which prevented the growth of the organism was that present in the last tube showing no color change.

In the procedure described by Fielding, the test organism and body fluid to be tested were mixed on a paraffined slide, and then taken up in capillary tubes following the technique of Fleming.<sup>2</sup> Because this technique is not commonly employed routinely in this country, it seemed advisable to attempt to utilize the same principle and devise a micromethod employing the more conventional procedures involved in serial test tube dilutions. The method to be described fulfills the following criteria: (1) It requires only that amount of blood which can be taken easily from a finger prick. (2) It uses an easily prepared test medium. (3) The end point is easily read by means of a sharp color change. (4) The procedure is one commonly employed in serology. (5) Finally, its accuracy appears to be superior to that obtained by the widely employed method described originally by Rammelkamp<sup>3</sup> and frequently modified.

### TECHNIQUE

*Collecting of Blood.*—The tube which was found convenient for drawing blood and separating the serum has been described previously<sup>4</sup> and is illustrated in Fig. 1.\*

Blood was taken from finger or heel puncture by capillary attraction and allowed to clot in the body of the tube. The tube was then filed and broken off above the level of the blood which was then centrifuged at 1,500 to 2,000 r.p.m. for ten to twenty minutes. The serum that separated was pipetted off for testing.

*Organism.*—The organism used in this laboratory was *Staph. aureus* (F.D.A. strain 209). There is no reason why any penicillin-sensitive, easily cultivated organism could not

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\*Shell vials 95 by 7.5 mm. or glass tubing of 7.5 mm. bore and of convenient length (80 to 100 mm.) sealed at one end can be used. The open end is drawn out to capillary tip and sealed. A small hole is blown through the wall of the tube by heating it 25 mm. below the beginning of the capillary portion.

be used equally well. The organism, after a preliminary incubation at 37° C., was kept as a stock culture on plain nutrient agar (Difco) in a refrigerator. It was subcultured once a week onto fresh agar. When required for the test, the organism was transferred to nutrient broth (Difco) and incubated at 37° C. for eighteen hours. A portion was then diluted 1:1,000 in the assay medium to be described below.



Fig. 1.

*Medium.*—The assay medium was phenol red dextrose broth made up as follows:

Nutrient broth (Difco)	8 Gm.
Dextrose	5 Gm.
Aqueous phenol red	25 ml.
Distilled water	1,000 ml.

This was adjusted to pH 8 with N sodium hydroxide using the phenol red (0.04 per cent concentration) as indicator, the final color being red. After autoclaving for fifteen minutes at 15 lb., the medium was ready to use without further adjustment of the pH.

*Procedure.*—The test was arranged so that one-half step dilutions were used, and a triplicate penicillin control test was included with each series of unknowns.

*Control.*—This was included routinely to detect the sensitivity of the test organism. A penicillin solution was made up in the assay broth in such a way that the penicillin concentration was 0.5 unit per milliliter. The one-half step dilutions were made as follows. Three sets, each containing thirteen plugged, sterile Wassermann tubes (100 by 13 mm.), were arranged in suitable racks. In each set of tubes, the following steps were carried out. The test medium (phenol red dextrose broth) was added in 0.1 ml. amounts to tubes 1, 3, and 4 to 12 inclusive. Tube 2 was left empty, and 0.2 ml. of the test medium was added to tube 13. The penicillin solution was then added in 0.1 ml. amounts to tube 1 which made a 1:2 dilution and to tube 13 which made a 1:3 dilution. Tube 1 (1:2 dilution) was then used after thorough mixing, for preparing one series of twofold (one step) dilutions by transferring 0.1 ml., serially to the odd numbered tubes 3 to 11, 0.1 ml. from tube 11 being discarded. From tube 13, which contained 1:3 dilution of penicillin, 0.1 ml. was added to the empty tube 2 (1:3 dilution) and to tube 4 which already contained 0.1 ml. assay broth (1:6 dilution). Tube 13 was then discarded. Tube 4, after thorough mixing, was used to initiate the second series of twofold (one step) dilutions by transferring 0.1 ml. serially to the even numbered tubes 6 to 12, 0.1 ml. from tube 12 being discarded. Tubes 1 to 12 in each set then contain 0.1 ml. of assay medium, with concentrations of penicillin decreasing from 1:2 to 1:96 by one-half steps.

*Test Fluids.*—A maximum of 0.2 ml. was required for each test fluid. Except that only one set of tubes was run, this was handled in a similar manner to that just described, the fluid taking the place of the known penicillin solution.

*Test Organism.*—0.1 ml. of the 1:1,000 dilution of the eighteen-hour culture of the test organism described was then added to every tube and the complete series incubated overnight at 37° C.

*End Point.*—Since, with this indicator, a change from red to yellow indicates a lowering of pH resulting from bacterial growth, the end point was taken as the last tube in each set in which the original red color of the medium was unaltered. This end point was usually quite sharp. Since the controls were set up in triplicate, it sometimes occurred that the end point varied somewhat in one of the three sets, although this variation was seldom more than one-half step off in either direction. The end point taken was that reached in two of the three control sets. If there was no such agreement, the test was considered invalid.

*Calculation.*—First, the sensitivity of the organism was calculated from the controls. Since the concentration of penicillin in the first tube of each control series was 0.5 unit per milliliter, the concentration in any subsequent tube could be calculated by dividing 0.5 unit per milliliter by the reciprocal of the dilution of that tube. For example, if the end point in two of the three control series was found to be tube 7 or a 1:16 dilution, then the organism was sensitive to a concentration of  $\frac{0.5}{16}$  or 0.03 unit per milliliter.

The concentration of penicillin in any unknown body fluid was easily calculated by multiplying the sensitivity of the organism, as determined in the preceding paragraph, by the reciprocal of the dilution of body fluid contained in the end point tube. For example, if in the test the sensitivity of the organism was 0.03 unit per milliliter and the end point of the body fluid being tested was found to be tube 5 or 1:8 dilution, then the concentration of penicillin in the original body fluid was  $0.03 \times 8$  or 0.24 unit per milliliter.

In the laboratory it was found convenient to record the dilution and the end points of the controls and the test fluids on squared paper. This avoided any mistake in the dilutions (Table I).

TABLE I. SAMPLE CHART FOR MICRO-PENICILLIN ASSAY

TUBE	1	2	3	4	5	6	7	8	9	10	11	12	PENICILLIN (UNITS/ML.) DETECTED
DILUTION	2	3	4	6	8	12	16	24	32	48	64	96	
PENICILLIN (UNITS/ML.)	0.25	0.17	0.125	0.085	0.065	0.04	0.03	0.02	0.015	0.01	0.0075	0.005	
Control 1							x						0.03
2							x						
3						x							
Pt. 1										x			0.96
1 1/2 hr.							x						0.36
3 hr.				x									0.12
Pt. 2											x		1.44
1 1/2 hr.								x					0.48
3 hr.					x								0.18
Pt. 3								x					0.48
1 1/2 hr.								x					0.48
3 hr.						x							0.24

*Accuracy of the Method.*—This method was first tested on five different occasions by attempting on each occasion to determine the amount of penicillin in each of five twofold serial dilutions of a known concentration of penicillin in distilled water. Table II shows the results obtained by the method just described. It can be seen that the recoveries at each dilution were remarkably close to the amount of penicillin actually present. The average of the tests was  $\pm 8$  per cent, although individual readings sometimes deviated as much as 44 per cent. Comparison with the results obtained by the Rammelkamp method

TABLE II. DETERMINATION OF ACCURACY OF MICROMETHOD OF PENICILLIN ASSAY

KNOWN PENICILLIN CONCENTRATION	UNITS/ML. OF PENICILLIN IN SERUM				
	0.1	0.5	1.0	2.0	4.0
Test 1	0.12	0.48	1.08	1.92	3.84
Test 2	0.12	0.72	1.08	1.92	3.84
Test 3	0.12	0.48	1.08	1.92	3.84
Test 4	0.12	0.72	0.72	1.92	3.84
Test 5	0.09	0.48	1.08	1.92	3.84
Average	0.11	0.57	1.0	1.92	3.84

(Table III) indicates that the micromethod was more accurate. However, there appeared to be a definite relationship between the two, the micromethod giving a consistently higher reading by a factor of 1.6. Fig. 2 shows a graphic comparison of the known concentrations of penicillin and the recoveries by the two methods used.

TABLE III. DETERMINATION OF ACCURACY OF THE MODIFIED RAMMELKAMP METHOD OF PENICILLIN ASSAY

KNOWN PENICILLIN CONCENTRATION	UNITS/ML. OF PENICILLIN IN SERUM				
	0.1	0.5	1.0	2.0	4.0
Test 1	0.052	0.3	0.832	1.22	2.43
Test 2	0.052	0.3	0.832	1.22	2.43
Test 3	0.052	0.3	0.624	1.22	2.43
Test 4	0.052	0.3	0.624	1.22	2.43
Test 5	0.052	0.3	0.624	0.91	2.43
Average	0.052	0.3	0.71	1.15	2.43

COMPARISON OF MODIFIED FIELDING AND RAMMELKAMP METHODS OF PENICILLIN BIO-ASSAY

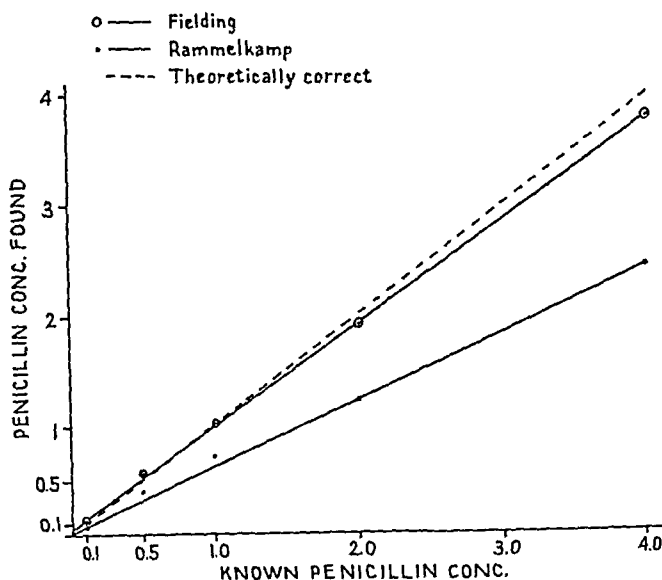


Fig. 2.

Following the demonstration that known amounts could be assayed with a good degree of accuracy, this test has been used successfully for hundreds of routine assays of penicillin levels in body fluids.

#### SUMMARY

A micromethod for the estimation of penicillin levels in blood or other body fluids, requiring only 0.2 ml. for the test, is described. Sufficient blood can be collected conveniently from a single puncture wound of the finger. A convenient collecting tube for obtaining serum is described and illustrated.

The principle employed in the test is the detection of growth of the test organism in a glucose-containing medium by the demonstration of acid formation by the color change of a suitable indicator. The end point is taken as the last tube to show no evidence of color change. By including controls in each test, the quantity of penicillin in an unknown penicillin fluid can be calculated. The accuracy of the method is greater in our hands than the commonly employed Rammelkamp method, and it has proved a convenient assay method for routine use in children.

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# THE PRODUCTION OF ANTIRABBIT HEMOLYSIN

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THE hemolytic system most commonly used in complement fixation tests consists of sheep erythrocytes and antish sheep hemolysin. A supply of fresh sheep erythrocytes for the performance of the test necessitates constant maintenance of a few sheep on the premises, which is often inconvenient. Attempts have been made by Vedder<sup>1</sup> and by me<sup>2</sup> to overcome this difficulty by a different hemolytic system. The object of the present communication is to describe another modification, in which the role of rabbit and sheep is reversed. Rabbit erythrocytes are employed with an antirabbit hemolysin of the sheep.

## METHODS AND MATERIALS

Five sheep were selected for this study, two adult males, two adult females, and one female lamb.

To each 50 ml. of freshly drawn rabbit blood about 2 ml. of 0.4 per cent sodium citrate were added to prevent clotting. The erythrocytes were washed three times in sterile 0.9 per cent NaCl solution and the packed red blood corpuscles were resuspended in equal volume of saline for inoculum.

Hemolyzed rabbit red blood corpuscles and the stroma of rabbit erythrocytes as prepared by Vedder were tried out as an alternate inoculum.

The inoculations were either intravenous or subcutaneous or both, and the dose for each inoculum varied from 5 ml. to 200 milliliters. Control blood samples were taken from the sheep before initial inoculations. Samples were taken also at the end of each course of immunization and after inactivation at 56° C. for thirty minutes they were tested for hemolysin.

Antirabbit hemolysin titrations were made with 2 per cent washed rabbit erythrocytes. In some of the tests, titrations were made with blood taken from the same rabbits that had been bled previously for immunization of the sheep.

Pooled guinea pig complement in dilutions of 1:15 and 1:30 was used in the hemolysin titrations. The titration was checked by using the standard Kolmer hemolysin titration test with antish sheep hemolysin and 2 per cent sheep red corpuscles.

Wassermann-positive and Wassermann-negative human sera were tested with the antirabbit hemolysin and 2 per cent washed rabbit erythrocytes by the modified Kolmer method and with the standard Kolmer-Wassermann complement fixation test, Kolmer,<sup>3</sup> and the results were compared.

## EXPERIMENTAL DATA

The five sheep used in this study differed in their production of antirabbit hemolysin.

The sheep which were inoculated with large doses of rabbit erythrocytes at frequent intervals produced hemolysin promptly and in higher titer as compared with the sheep which were inoculated with smaller doses of erythrocytes,

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even when inoculations were spread over a longer period of time. When sheep were immunized for hemolysin, it was noted that agglutinin for rabbit erythrocytes occurred much sooner and in higher titer than did antirabbit hemolysin, particularly when the period of immunization was prolonged. Thus Sheep A at the end of seventeen days had no hemolysin while the agglutinin titer was 1:2,400. The same sheep at the end of ninety-three days of immunization had a hemolysin titer of 1:30 and the agglutinin titer was 1:10,240.

In Sheep D and E, which were immunized in massive doses and at frequent intervals, agglutinin and hemolysin occurrence were almost simultaneous.

The results of the production of antirabbit hemolysin in sheep are summarized in Table I.

Sheep A (male), which was immunized with small quantities (5 to 10 ml.) intravenously of washed rabbit erythrocytes, produced hemolysin after sixty-nine days of immunization; however, during the entire course of immunization (ninety-three days), hemolysin titer never exceeded 1:30.

Sheep B (male) received sixty-nine injections totaling 250 ml. of lysed rabbit erythrocytes and 2,455 ml. of washed rabbit erythrocytes in a period of 290 days. The immunization of this sheep was begun with small amounts (10 ml.) of hemolyzed red blood corpuscles. During fifty days of immunization, this sheep was inoculated nineteen times with 190 ml. of hemolyzed erythrocytes. At the end of this period, the hemolysin titer was only 1:4. Since the lysed corpuscles failed to produce a significant hemolysin titer and inasmuch as Sheep A already had produced hemolysin following injection of intact erythrocytes, at this stage the inoculation of lysed erythrocytes was discontinued. Subsequent inoculations with rather large doses (25 to 100 ml. per inoculum) of intact erythrocytes increased the hemolysin titer to 1:80. Prolongation of immunization, instead of increasing, decreased the hemolysin titer to 1:20 (see Table I).

Sheep C (female) received thirty injections totaling 1,400 ml. of washed rabbit erythrocytes and 110 ml. of stroma of rabbit erythrocytes during a period of 234 days. The immunization of this sheep was begun with rather large doses of washed rabbit erythrocytes and the highest hemolysin titer obtained was 1:20. Since the sheep manifested mild anaphylactic symptoms, intravenous inoculations were substituted with stroma. In spite of further immunization with stroma, which subsequently was followed with intact erythrocytes, the hemolysin titer did not increase (see Table I).

Sheep D (pregnant female) was immunized with fifty-four injections totaling 4,200 ml. of washed rabbit erythrocytes in a 212-day period. The immunization of this sheep was done with massive doses and at frequent intervals. Hemolysin with titer of 1:40 was present as early as twenty-three days following initial inoculation. While the sheep was undergoing immunization, it gave birth to a lamb. Four days after the lamb was born, blood samples taken from the sheep showed hemolysin titer of 1:320. Subsequent inoculations with 50 and 100 ml. of washed erythrocytes were at times accompanied with slight to moderate anaphylactic reactions such as sneezing, coughing, discharge of feces and bloody urine, weakness, inability to move, salivation, tremors, and dyspnea.



TABLE I. THE PRODUCTION OF ANTIRABBIT HEMOLYSIN IN SHEEP

SHEEP	INOCULA- TION	ERYTHROCYTES		HEMOLYZED ERYTHROCYTES		STROMA		ROUTE OF INOCULATION	DAYS BETWEEN EACH INOCULA- TION	REST PERIOD (DAYS)	SEROLOGIC TESTS	
		ML. PER EACH INOCULA- TION	TOTAL ML.	ML. PER EACH INOCULA- TION	TOTAL ML.	ML. PER EACH INOCULA- TION	TOTAL ML.				DAYS AFTER FIRST INOCULA- TION	HEMOLY- SIN TITER
A	1-9	5	45	-	-	-	-	I.V.	2 to 3	26	17	0
	10-18	10	90	-	-	-	-	I.V.	2 to 8	3	69	1:24
	19-27	10	90	-	-	-	-	S.C.	1 to 5	3	93	1:30
B	1-10	-	-	10	100	-	-	I.V.	2 to 4	3	26	0
	11-19	-	-	10	90	-	-	S.C.	2 to 5	3	50	1:4
	20-22	-	-	5-40	60	-	-	I.V. and S.C.	3	4	-	-
	23-29	25	175	-	-	-	-	S.C.	4 to 5	5	96	1:40
	30-40	50	550	-	-	-	-	I.V.	3 to 6	2	140	1:80
	41-46	50	300	-	-	-	-	I.V.	1 to 3	2	-	-
	47-52	50	300	-	-	-	-	S.C.	Daily	4	161	1:80
	53-60	40-70	380	-	-	-	-	I.V.	2 to 3	20	218	1:40
	61-66	100	600	-	-	-	-	I.V.	2 to 3	3	239	1:20
	67-69	50	150	-	-	-	-	I.V.	1 to 3	26	290	1:20
C	1-7	50	350	-	-	-	-	I.V.	3 to 4	2	23	1:10
	8-14	50-100	600	-	-	-	-	I.V.	2 to 4	146	44	1:20
	15-21	-	-	-	-	5-8	70	I.V.	2 to 11	4	-	-
D	22-30	50	450	-	-	8	40	S.C. and I.V.	1 to 2	30	233	1:10
	1-10	50	500	-	-	-	-	I.V.	2 to 3	2	23	1:40
	11-18	50-100	700	-	-	-	-	I.V.	2 to 3	21	45	1:320
	19-25	50-100	700	-	-	-	-	I.V. and S.C.	2 to 3	3	77	1:80
	26-28	200	600	-	-	-	-	I.V. and S.C.	2	24	86	1:80
	29-35	100	700	-	-	-	-	I.V.	2 to 3	24	122	1:40
	36-38	50	150	-	-	-	-	I.V.	1 to 3	30	173	1:80
E	39-50	50	600	-	-	-	-	I.V.	2 to 3	4	204	1:40
	51-54	50-100	250	-	-	-	-	I.V. and S.C.	1 to 3	-	212	-
	1-7	100-200	800	-	-	-	-	I.V.	2 to 3	24	17	1:10
	8-10	50	150	-	-	-	-	I.V.	1 to 3	30	68	1:20
	11-16	50	300	-	-	-	-	I.V.	2 to 3	2	85	1:10
	17-22	50	300	-	-	-	-	I.V.	2 to 3	4	99	1:10
	23-35	50-100	750	-	-	-	-	S.C. and I.V.	1 to 12	30	137	1:20
	36-40	30-50	210	-	-	-	-	S.C.	2 to 3	12	180	1:10

In spite of continuation of immunization, the hemolysin titer never exceeded 1:320, but diminished to 1:40. During the last intravenous inoculation, the sheep died of anaphylactic shock.

Sheep E. This female lamb, born to immunized Sheep D, was tested at the age of 2 months for the presence of possible antirabbit hemolysin, with negative results. This was followed by forty injections of rabbit red blood corpuscles, totaling 2,510 ml. during 180 days. The first seven inoculations were made intravenously in 200 ml. amounts every other day. The highest hemolysin titer obtained at various stages of immunization was 1:20 (see Table I).

Antirabbit hemolysin with a titer of 1:40 or above and a 2 per cent suspension of washed rabbit erythrocytes was tested with Wassermann-positive and Wassermann-negative human sera by Kolmer technique; because of the low hemolytic titer of antirabbit hemolysin, relatively large amounts of this hemolysin were used. As a control, parallel tests were made with Kolmer-Wassermann method. Forty-two syphilitic and forty-two normal sera were run by each method. The results of this test were clear-cut, and known positive samples gave positive readings and negative samples gave negative readings by both procedures. The findings by the experimental method were identical with those obtained with antish sheep hemolysin and washed sheep erythrocytes, as used in the usual Wassermann test.

#### SUMMARY

Antirabbit hemolysin was produced in sheep by inoculating them intravenously and subcutaneously with washed rabbit red blood corpuscles. The hemolysin titer acquired by these animals ranged from 1:10 to as high as 1:320.

Hemolyzed erythrocytes and stroma injected into sheep for the production of hemolysin did not produce as high a titer as washed erythrocytes.

Antirabbit hemolysin and 2 per cent washed rabbit erythrocytes were used in testing syphilitic and normal sera by modified Kolmer-Wassermann technique. The results obtained in every instance were clear-cut and of diagnostic value.

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# DIFFERENTIATION AND ENUMERATION OF EOSINOPHILS IN THE COUNTING CHAMBER WITH A GLYCOL STAIN; A VALUABLE TECHNIQUE IN APPRAISING ACTH DOSAGE

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THE decrease in the circulating eosinophils following the intramuscular injection of adrenocorticotrophic hormone (ACTH, Armour) was first described by Hills, Forsham, and Finch<sup>1</sup> and subsequently by Hellman.<sup>2</sup> The increasing importance of ACTH in the diagnosis<sup>3</sup> and treatment<sup>4, 5</sup> of various clinical conditions as well as in other clinical studies of the effects of this hormone<sup>6, 7</sup> underscores the importance of a simple, reliable technique for enumerating eosinophils, as the level of these cells in the peripheral blood may be a general index of the sensitivity of the gland stimulation and may be a general guide to adequacy of dosage in a given case.<sup>8, 9</sup>

In this connection the merits of a white blood cell diluting fluid of equal parts propylene glycol and water containing phloxine and methylene blue as stains should be re-emphasized because it is the simplest and most accurate method of enumerating blood eosinophils. This point is important because the number of circulating eosinophils is a general guide of adequate dosage of ACTH in treating various disease syndromes. Although our own observations in this respect† are not ready for publication, a review of the technique applied in enumerating eosinophils seems indicated because of the rapidly increasing medical interest in adrenocorticotrophic hormone (ACTH).

Hypotonic diluting fluids of the type originally described by Dunger<sup>10</sup> or as modified by Hills, Forsham, and Finch<sup>1</sup> and Thorn and associates<sup>3</sup> are not entirely satisfactory for counting eosinophils in the peripheral blood. This statement is based on the experience of employing Camara and Alvarez<sup>11</sup> modification of Dunger's diluting fluid consisting of 5 parts of 1 per cent aqueous eosin, 5 parts acetone, and 100 parts distilled water for a period of three years in studying cases of food and drug allergy. The staining fluid used by those studying the effects of ACTH is identical except that 2 per cent instead of 1 per cent aqueous eosin was employed.

The inadequacies of this type of diluting fluid were first pointed out in 1944.<sup>12</sup> It was determined that the counting chamber estimation of eosinophils by means of hypotonic diluting fluids containing eosin as modified from Dunger's original description remains subject to considerable error in that the ruptured or "ghost" cells are identified by clumps of eosin-staining granules retained in portions of the cell membrane. In 1947 it was again emphasized by me<sup>13</sup> that the use of such diluents was fraught with considerable error in that one frequently encountered indeterminate forms that could not with certainty

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be classified as eosinophils. In addition, a greater absolute number of eosinophils usually was obtained from employing this type of hypotonic diluent than was determined from performing parallel stained film differential counts. It was further pointed out, however, that the errors inherent in this method became less significant if the determinations were made with a short but constant time interval elapsing between the dilution of the sample and the completion of the counting. These observations have recently been confirmed by Henneman, Wexler, and Westenhaver.<sup>14</sup> In their experience with the Dunger technique as modified by Thorn and associates, there was a large and rapid decrease in the eosinophil cell count with passage of time after dilution of the blood and with ageing of the oxalated blood. Because of this and other reasons, these authors were unable to recommend the use of this type of diluent.

The so-called "hemolytic" and solvent properties of propylene glycol suggested its use as a white cell diluting fluid and staining base in 1943.<sup>15</sup> Upon closer observation it was learned that the apparent hemolytic action of the glycols, as reported by Von Oettingen and Jirouch,<sup>16</sup> was not a true hemolysis, but that the disappearance of human erythrocytes suspended in equal parts propylene glycol and water was due to the fact that the red blood cells rapidly assumed the same density as the surrounding media and thus became relatively nonrefractile. This phenomenon was studied in more detail by Randolph and Mallery<sup>17</sup> who were able to demonstrate that a mixture of human blood in equal parts propylene glycol and water appearing to be hemolyzed as viewed grossly in a test tube or under the light microscopic field actually was not hemolyzed when viewed through the dark-field microscope. Under these circumstances the erythrocytes retained their normal contours.

The technique described in 1944<sup>12</sup> and employed in subsequent studies consists of the following:

Given amounts of stock solutions containing 0.1 per cent methylene blue in propylene glycol and 0.1 per cent phloxine in propylene glycol are each diluted with an equal volume of distilled water and placed in dropper bottles. For example:

Solution I*		
0.1 per cent methylene blue in propylene glycol	50.0 c.c.	
Distilled water	50.0 c.c.	
Solution II*		
0.1 per cent phloxine in propylene glycol	50.0 c.c.	
Distilled water	50.0 c.c.	

The final white blood cell diluting fluid is made by mixing an equal number of drops of Solution I and Solution II in a test tube; this remains usable for approximately four hours. After standing for longer periods, differential staining detail may become impaired and precipitation of dyes may occur. Curiously, prior to four hours the two dyes do not seem to mix completely as shown by the ability of the phloxine to disperse in a filter paper at a greater rate of speed than does the methylene blue, a drop on such a medium separating as to color and leaving a blue center and a red periphery in a manner akin to the principle employed in paper chromatography.

\*As has been pointed out,<sup>17</sup> the effect of propylene glycol on erythrocytes as far as the timing of color and due to summer the leucocyte count is dependent upon concentration of the glycol and the amount of water from the stock or working solutions. The effect of dilution of propylene glycol to the point that in before one has an opportunity to enumerate solutions containing 40 or 45 per cent propylene glycol.

Because of the variation in staining ability between lots of dyes, optimum acid and basic staining may sometimes be obtained by making a slight change in the relative proportions of Solution I and II in the final diluent. For example, four parts of Solution I and six parts of Solution II might result in better staining technique than if equal parts of the two were mixed.

Under certain circumstances the phloxine tends to crystallize out in long narrow crystals from Solution II; this may occur after the solution has stood several weeks and may be remedied readily by simple filtration or by preparing Solution II from the stock at monthly intervals.

Experience indicates that it is helpful to clean the pipettes with agents other than acids or to be certain that acid radicles are completely removed from the interiors of the pipettes as a result of repeated rinsing. Cleaning the pipettes with water and drying by a final rinse with acetone and avoiding the use of a series of bottles also employed in cleaning pipettes containing acid diluents aids in avoiding complications in the use of this technique.

Total and differential white blood cell counts are made using the standard pipette and counting chamber. The pipette is shaken during the course of and immediately after filling. If the chamber is charged immediately after diluting the blood and shaking, a period of from three to five minutes is required for the "disappearance" of the red blood cells and settling of the leucocytes prior to determining the total white cell count. A total period of from ten to fifteen minutes is recommended for maximum staining of eosinophils and other leucocytes. Blood may be left in the pipette for varying periods, including overnight, without a significant change in the staining qualities of the cells or in the total leucocyte count, an obvious advantage over the use of hypotonic diluents employed in enumerating eosinophils.

Under the low magnification (16 mm. objective) eosinophils may be differentiated from other cells by their brilliant red color and slightly larger size. The contrast in color is intensified by increasing the light and raising the condenser of the microscope. The eosinophils in one side of the counting chamber (0.9 c.mm.) or in the entire ruled areas of both sides of the chamber (1.8 c.mm.) are counted and multiplied, respectively, by 22 and 11 to obtain the number per cubic millimeter of blood.

The differentiation and enumeration of polymorphonuclear and mononuclear cells are accomplished to best advantage by the use of high magnification (4 mm. objective and 15 $\times$  eyepiece). The first 100 cells encountered are so classified.

In this diluting fluid all white blood cells remain intact, round in contour, and relatively large in size. The granules of the eosinophils stain brilliant red, the nuclei of all cells aquamarine; this permits the differentiation of eosinophils from other white blood cells even in the presence of highly fragile leucocytes as observed in leucemia and other blood disorders.<sup>18</sup>

The increased viscosity of this medium is advantageous in that the end point in filling the pipette may be reached accurately, there is little settling and clumping of white cells, and there is no tendency toward overflow in charging the chamber. The clear background of the counting chamber and the relatively slow rate of evaporation from it are also advantageous.

The differentiation of eosinophils in the same sample of blood employed in determining the total leucocyte count results in a minimum possible error in comparison with alternative methods.

Finally, the glycol-stain technique also permits the counting chamber enumeration of polymorphonuclear and mononuclear leucocytes even in the presence of many young myeloid elements as observed in the course of acute allergic reactions following the ingestion of allergenic drugs.<sup>10</sup>

The accuracy of the counting chamber enumeration of eosinophils on one side of the chamber (0.9 c.mm.) using the propylene glycol technique as compared with the standard method of enumerating eosinophils, that is, by determining their number as a result of performing 200 cell film differential counts and applying the percentage obtained to the total leucocyte count, was studied by Randolph and Stanton,<sup>20</sup> employing parallel observations from the same freely bleeding puncture wound. Such determinations were made in 100 normal persons and in 400 instances in allergic individuals who had varying degrees of eosinophilia. Finally, ten of the latter subjects were subjected to ten parallel eosinophil counts each by the two techniques.

Table I lists the maximum, minimum, mean, and standard deviation determined as a result of the statistical analysis of these ten observations per subject by each of the two techniques.

TABLE I. EOSINOPHIL COUNTS AS DETERMINED IN ENUMERATING THE EOSINOPHILS IN ONE SIDE OF THE COUNTING CHAMBER (0.9 C.M.M.) COMPARED WITH THE DIFFERENTIAL ENUMERATION OF EOSINOPHILS FROM PERFORMING 200 CELL DIFFERENTIAL COUNTS FROM THE STAINED FILM

SUBJECT	EOSINOPHILS IN 0.9 C.M.M. OF CHAMBER WITH THE PROPYLENE GLYCOL TECHNIQUE				EOSINOPHILS FROM 200 CELL FILM DIFFERENTIAL COUNTS			
	MAX.	MIN.	MEAN	S. D.	MAX.	MIN.	MEAN	S. D.
Ch.	110	44	68	27.6	113	0	61	46.0
H.	710	422	562	83.4	722	420	553	100.0
G.	799	555	673	92.0	1,241	453	707	222.2
F.	799	666	726	53.6	1,278	402	676	229.4
M.	977	888	932	39.8	1,101	591	785	153.0
Q.	1,085	755	951	117.3	1,155	532	849	204.0
Co.	1,379	755	1,097	203.0	1,411	981	1,204	135.5
A.	2,065	1,121	1,461	303.2	1,937	1,061	1,448	249.2
E.	1,709	1,332	1,474	158.2	1,723	1,003	1,397	234.5
B.	1,865	1,221	1,543	197.3	2,218	1,484	1,850	216.0

From these studies it was determined that the enumeration of eosinophils on one side of the counting chamber (0.9 c.mm.) was a more accurate method of determining the number of these cells than the result of a differential count of 200 leucocytes from the stained film, employing the cover slip technique and a standard method of sampling the stained area.

Henneman, Wexler, and Westenhaver<sup>14</sup> recently compared the effectiveness of phloxine in equal parts propylene glycol in water (Solution II of this combined stain) with Thorn's modification of Dunger's hypotonic diluting fluid in enumerating eosinophils. In their experience with these two chamber techniques, the diluent composed of phloxine in propylene glycol not only provided more consistent results and no evidence of destruction of eosinophils but

also was preferred by them because of the ease in identification of cells, decreased evaporation of the diluent from the counting chamber, greater reproducibility of results, and because no refrigeration of the diluent was required. They confirmed our experience in using the cosin-acetone hypotonic diluent, commenting specifically on the rupturing of cosinophils in this medium and the large and rapid decrease in cell count with passage of time after dilution of the blood and with ageing of the oxalated blood.

Because of the more accurate enumeration of cosinophils permitted with the use of the glycol counting chamber stains, either the phloxine part of the diluent alone or the combined stain diluting fluid is preferred over alternative methods in following the levels of blood cosinophils in patients under treatment with adrenocorticotrophic hormone (ACTH).

#### SUMMARY

A white blood cell diluting fluid of phloxine and methylene blue dissolved in equal parts propylene glycol and water permits the counting chamber differentiation of eosinophils on the same blood specimen used in determining the total leucocyte count. In this medium the eosinophils stain bright red and are readily differentiated in the counting chamber from other polymorphonuclear and mononucleated leucocytes. This technique is the most accurate method of determining the number of eosinophils in the peripheral blood in that it permits the enumeration of intact stained cells in contrast to the previously described counting chamber techniques employing hypotonic diluents in which ruptured or "ghost" eosinophils are counted. It is a particularly valuable method to use in following the clinical course of patients under therapy with adrenocorticotrophic hormone (ACTH), those suffering from acute allergic reactions or infections in which one is primarily interested in the variations in serial determinations of the peripheral blood eosinophils.

This direct counting chamber technique makes it possible for the first time to express accurately the number of eosinophils existing in the peripheral blood; the expression of the number of eosinophils per cubic millimeter of blood is obviously more accurate and meaningful than to consider eosinophils in terms of the relative percentage of the total leucocyte count.

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# WARBURG MANOMETER CALIBRATOR\*

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## INTRODUCTION

IN CALCULATING the constant for a Warburg manometer it is necessary to know the volume of the Warburg vessel plus the volume of the manometer to the 150 mm. mark. To determine the latter volume the manometer is usually filled with mercury to the 15 cm. mark and the volume is determined from the weight and density of mercury. This method of calibration is very time consuming since it is somewhat difficult to introduce the requisite amount of mercury to just fill the manometer to the 15 cm. mark. The manometer calibrator herein described greatly simplifies and shortens the calibration procedure. The principle employed is similar to that used in the construction of the Scholander microburette.†

## CONSTRUCTION OF CALIBRATOR

The apparatus consists of a metric micrometer (Fig. 1, *A*) which is calibrated in hundredths of a millimeter. A stainless steel plunger (*B*) with a cross sectional area equal to 1 sq. cm. (1.000) is pressed onto the micrometer spindle (*C*). A stainless steel collar (*D*) is pressed over the end of the micrometer. The upper end of this collar is threaded to accommodate the Lucite top (*E*). This plastic top is turned to a standard taper joint at *H* so that it fits the conventional Warburg manometer. A doughnut shaped "O" ring (*F*) fits over the plunger (*B*) and is compressed between the plastic (*E*) and the collar (*D*). This "O" ring seals the plunger within the plastic, yet it permits advancement of the plunger into the space (*G*).

## METHOD OF CALIBRATION

In use, the plastic cylinder is filled with water and the spindle is nearly withdrawn from the chamber (*G*). The cleaned manometer is inserted in the standard taper (*H*) of the plastic and fixed in place with rubber bands (Fig. 2). It is desirable to have just sufficient water in the calibrator chamber (*G*) so that only a small air bubble remains within the plastic cylinder when the manometer is in place. The manometer is held in the vertical position and the stopcock (*M*) of the manometer is opened and the other ends of the manometer are sealed by rubber tubes (*N*) and pinch clamps (*O*). The micrometer plunger is advanced and all the air is carefully expelled from the calibration chamber (*G*). The water meniscus is adjusted to coincide with the calibration mark of the manometer (*J*) (Fig. 2) (i.e. the mark used in calibrating the vessels) and the micrometer scale is read. The manometer is now turned to a horizontal position

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\*Manufactured by Micrometric Instrument Company, 7929 Kinsman Ave., Cleveland 4, Ohio.

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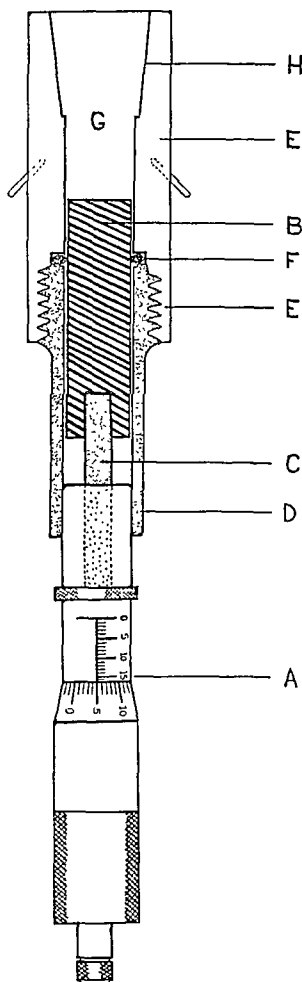


Fig. 1.—Construction of Warburg manometer calibrator (for explanation see text).

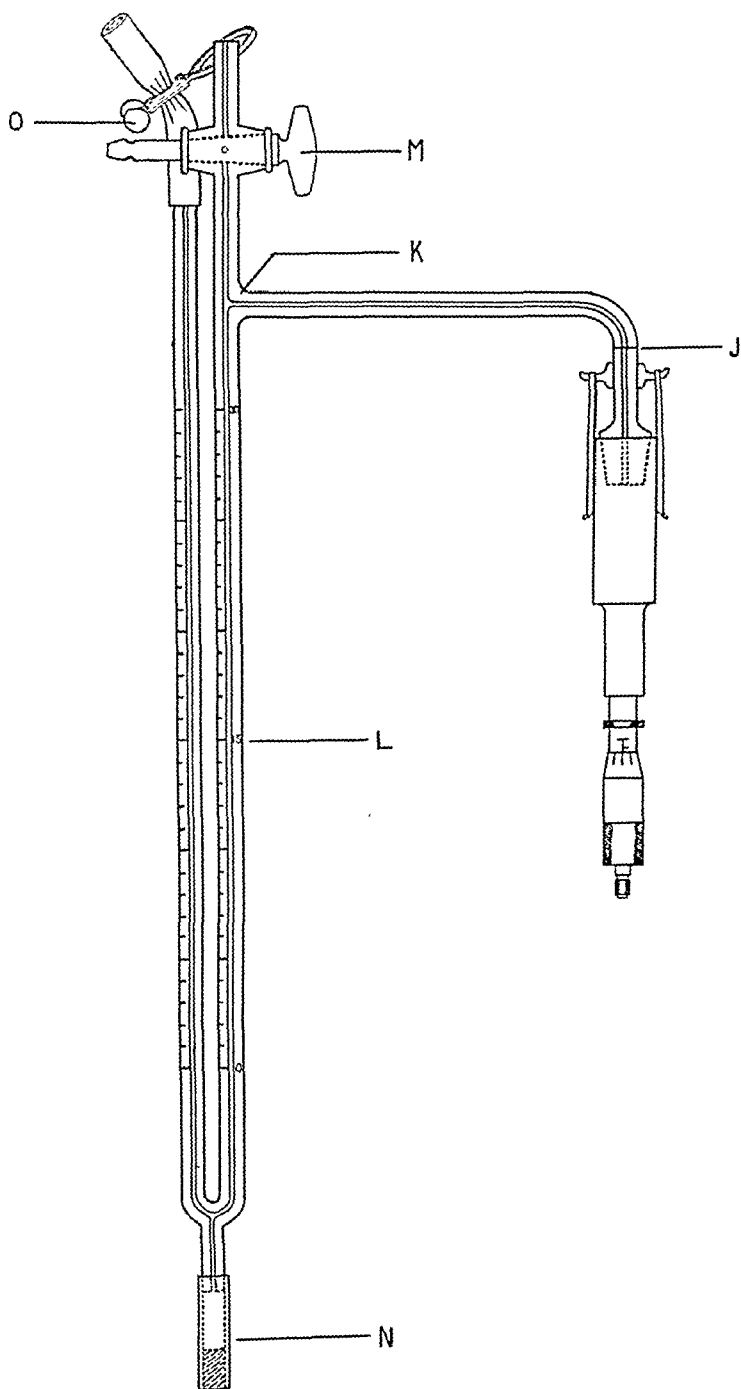


Fig. 2.—Calibrator inserted on Warburg manometer (for explanation see text).

and the plunger is advanced until the water meniscus reaches point *K* (Fig. 2). Further advancement of the water meniscus fills the capillary between point *K* and the stopcock (*M*). When water reaches the bore of the stopcock (*M*), the stopcock is closed and the pinch clamp (*O*) is opened. The liquid is advanced to the 15 cm. mark (*L*) of the manometer. The micrometer is again read and the volume determined by subtracting the initial from the final reading. The liquid is then removed from the manometer by reversing the foregoing procedure and the calibration may be repeated.

Results of several calibrations are shown in Table I. It will be noted that the volume of the manometer as determined in the first trial is slightly greater than that observed in subsequent trials. This difference is due to the residual water which is left in the manometer after the first calibration. It is further

TABLE I. WARBURG MANOMETER CALIBRATION

REMARKS	TRIAL	VOLUME (C.C.)		
		23	28	34
Manometer dry	1	.746	.803	.839
Manometer wet	2	.720	.787	.820
Manometer wet	3	.723	.789	
Water left in manometers		.025	.014	.019

noted that repeated runs on a wet manometer differ by less than .005 cubic centimeter. The accuracy of the volume measurement obtainable with this calibrator far exceeds the accuracy required for the calculation of the manometer constant. The accuracy of the calibrator itself may be checked by inserting a 2 c.c. pipette through a one-holed rubber stopper and inserting the stopper into the standard taper (*H*) of the calibrator. Since the plunger has a cross-sectional area of exactly 1 sq. cm., each millimeter advancement of the micrometer plunger corresponds to 0.100 cubic centimeter. Since it takes two revolutions of the micrometer to advance the spindle 1 mm. and since there are fifty small divisions per revolution of the micrometer, each small division of the scale of the micrometer corresponds to a volume of 0.001 cubic centimeter.

## SUMMARY

A simple, rapid calibrator for the Warburg manometer is described.

## A NEWLY DEVELOPED ELECTROMAGNETIC FLOW METER

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THE general principle of an electromagnetic flow meter is not new. It was first developed by Kolin<sup>1</sup> in this country and independently by Wetterer<sup>2</sup> in Germany. Kolin<sup>3, 4</sup> developed both an A.C. model and a D.C. model; the latter was subsequently modified by Joehim.<sup>5</sup> These methods featured sleeves of various design which contained electrodes in positions so that when the sleeve surrounded a blood vessel, the electrodes were in contact with the outside surface of the blood vessel. While such devices possessed the advantage of not requiring incision into the blood vessel, they incurred the disadvantages of voltage drift and minor artifacts due to vessel drying, varying vessel diameter, and extraneous potential pickup. In order to avoid these disadvantages, a new method of potential pickup was developed in this laboratory which has proved exceedingly practical for the measurement of blood flow when used with an A.C. amplifier and suitable recorder. This newly developed electromagnetic blood flow meter has been found to be unusually accurate in its measurement of blood flow and to possess negligible voltage drift.

### PRINCIPLE

The basic principle upon which the operation of an electromagnetic flow meter depends is that when an electric conductor moves across the lines of force of a magnetic field, a potential difference is created in the conductor. If the field is uniform, the conductor moves in a plane at right angles to the magnetic field and the length of the conductor extends at right angles to both the field and the direction of motion; the resulting electromotive force will be directly proportional to field strength, speed of the conductor, and length of the conductor within the field. Therefore:

$$E = B \cdot l \cdot v \cdot 10^{-8}$$

where  $E$  is the potential difference in volts;  $B$ , field strength in gauss;  $l$ , length of the conductor within the field in centimeters (across the electrodes); and  $v$ , speed of the conductor in centimeters per second. By use of the A.C. system, including an A.C. magnet, there is an additional constant to be accounted for because of the electrodes and their leads acting as a one-turn transformer. Therefore, the total equation of an A.C. system must be altered to read as follows:

$$E = B \cdot l \cdot v \cdot 10^{-8} + A$$

where " $A$ " voltage is the potential developed by the electrodes and leads, this voltage being proportional to  $B$  field strength. In order to cancel out a portion of " $A$ " voltage, on the magnet a turn is made which is phased oppositely to that of the leads. The canceling potentiometer is turned in a manner to diminish " $A$ " voltage to a minimum. Once

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this point is established, the voltage is of a constant and easily accountable magnitude. If  $B$  and  $l$  are kept constant,  $K$  may be stated:

$$K = B \cdot l \cdot 10^{-9}$$

and

$$E = K \cdot v + A$$

As shown by the equations, when the voltage " $A$ " is subtracted, the electromotive force is a function of the single variable, the velocity of flow, which theoretically is linear. However, for practical considerations one is interested in volume flow in cubic centimeters per minute, rather than velocity flow in centimeters per second. These quantities are related as follows:

$$Q = 60 \cdot v \cdot \frac{2 \pi d^2}{4}$$

where  $Q$  is the volume flow in cubic centimeters per minute;  $v$ , the velocity flow in centimeters per second; and  $d$ , the diameter of the tube or vessel. While this formula shows the relations of quantities, it is not required for the use of the newly developed meter because, due to the manner of its construction, calibrations may be made of actual flow during an increment of time to read directly as cubic centimeters per minute blood flow.

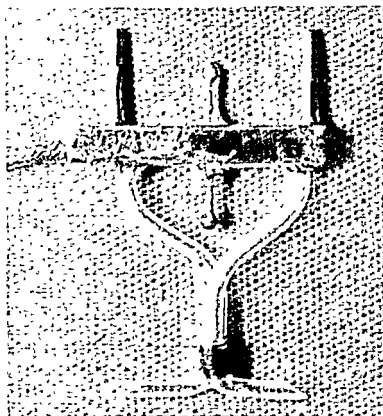


Fig 1.—Cannula used with electromagnetic blood flow meter.

#### MATERIALS AND METHODS

*Description of Apparatus.*—The complete flow meter consists of a specially constructed cannula, an A.C. magnet, an A.C. amplifier, and an Esterline-Angus 5 Ma. recorder.

The construction of the cannula consists of a glass or plastic tube of previously determined diameter to equal the size of the lumen of the blood vessel to be cannulated. The cannula and pickup shown in Fig. 1 has a diameter of 3 millimeters. This particular cannula which is designed to fit the femoral artery of a dog is 4 cm. in height and 2.5 cm. in width; the bottom forms an anchorage to secure ligatures. Fig. 2 better reveals the detailed construction. In the top of the cannula are inserted two tungsten (platinum may be used) wire rods with rounded tips so that the tips just touch the periphery of the

lumen. Onto these tips just outside of the glass are soldered the two pickup leads which are then tightly twisted and passed through the center of the housing and into a shielded cable which runs to the amplifier connection. On the top of the housing are two pins which fit into the magnet case in order to assure identical positioning of the cannula during each measurement.

The housing is filled with Lucite for the purpose of stabilizing the position of the lead wires in the magnetic field. This is done by dissolving a portion of Lucite in  $\text{CHCl}_3$ , pouring the solution in the housing around the cannula and leads, and letting it congeal to form solid Lucite. This operation also makes the housing repellant to liquids. The

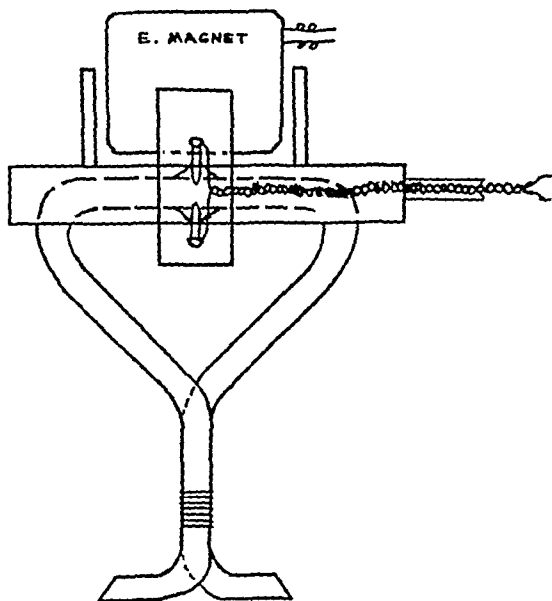


Fig. 2.—Diagram of cannula and pickup leads showing the position of the magnet.

slender base of the cannula is wrapped tightly with a single layer of No. 8 linen thread and coated with the liquid plastic to add strength to the cannula. Care is taken in the construction of the cannula that there are no constrictions in the lumen and that there are no sharp bends. The short section across the top which holds the electrodes is straight.

The cannula and pickup incorporates the following features: (a) an electrical system well isolated from the surrounding tissues; (b) a lumen of uniform size to match the size of the lumen of the blood vessel measured, with no constrictions in its length; (c) a minimum of glass or plastic surface (less than four inches in length) with which the blood is in contact; (d) a reinforced slender base around which to secure ligatures surrounding the ends of the arteries; and (e) a specially designed bronze housing surrounding the electrodes through which the tightly twisted pickup leads are inserted to traverse the center of the magnetic field and be shielded from contact with the magnetic poles. It is the design of this housing which allows a minimal and invariable background pickup and thus prevents voltage drift on the recorder.

The A.C. magnet used with the meter is made from stacked laminations of  $\frac{1}{2}$  inch 29 gauge L-4 audio A iron. The coil is made of 1,800 turns of No. 25 wire excited with a 110 V. A.C. 60 c.p.s. source. This gives a field of about 1,000 gauss across a  $\frac{1}{2}$  cubic inch air gap between pole tips. A 60 c.p.s. power source limits the frequency response of the whole system to fluctuations of less than 60 c.p.s., but it is possible to modify this limitation by changing the carrier frequency to 400 c.p.s. if desired. Should the meter be used

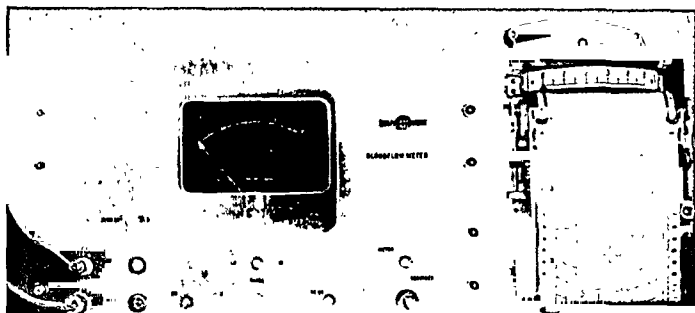


Fig. 3.—Recorder and amplifier showing the connections as labeled on the panel.

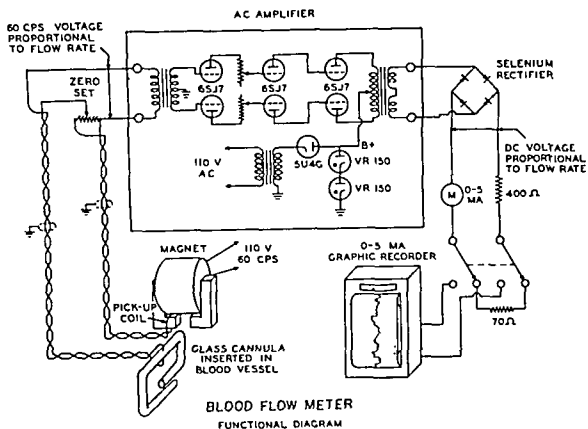


Fig. 4.—Simplified diagram showing the electromagnetic pickup, amplifier, and recorder.

with a high frequency response recorder, this modification is recommended. For convenience in affixing the position of the magnet, a screw-type lift with a crank attached is used to slowly lower the magnet into position over the cannula. The magnet is held by a ball-in-socket swivel which will allow it to be adjusted at a convenient angle.

The A.C. amplifier and recorder used are shown in Fig. 3. The amplifier is adapted from the original A.C. amplifier of Kolin, with minor modifications. Three stages of 6SJ7 tubes are used for the principal circuit with two VR 150 tubes being used on the B+ circuit.



This amplifier has proved remarkably stable and features two input channels for pickup, each connected to a potentiometer to be utilized in canceling out stray background. A selenium rectifier is used in the output circuit. Any stable amplifier with sufficiently high gain would be adaptable for the electromagnetic flow meter, so long as it matches the characteristics of the pickup and the magnet used. This particular amplifier is most adaptable to a 60 c.p.s. magnet. The amplifier used here has two gain settings, the two amplifications giving a total range of blood flow measurement from zero to 300 c.c. per minute with different cannulae. The range of measurement may be increased by constructing the

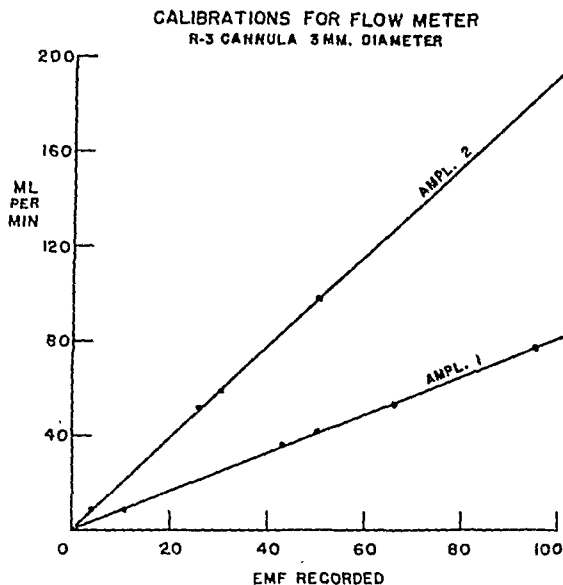


Fig. 5.—Sample calibration curve showing the use of two amplifications with one cannula.

electrodes of the cannula so that the distance between the tips is diminished. Conversely, greater sensitivity is achieved by constructing the electrodes of the cannula so that the distance between the tips is greater. In all cases the lumen of the cannula should be as nearly as possible identical with the size of the blood vessels.

A 5 Ma. Esterline-Angus recorder is used on the electromagnetic flow meter as a compromise choice of various possibilities. This instrument requires 5 Ma. through an internal resistance of 70 ohms for full scale deflection. This is a heavily damped instrument and will not record events accurately under 200 milliseconds but this feature is of value in the recording of mean blood flow where a concomitant record only of heart rate and respiration is desired. When events of shorter duration are of interest, a recorder of higher frequency response should be employed. Fig. 4 reveals a functional diagram of the entire apparatus as it is operated showing the position of the magnet, the general structure of the amplifier, and the recorder connections.

*Calibration of the Instrument.*—Although a fore-pump perfusion system has been used to calibrate the flow meter, it is felt that the preferred method is to measure the flow of blood out of a blood vessel directly into a graduate over a convenient period of time. This method is to be desired for its simplicity and the elimination of extraneous intervening variables. In operation, the distal end of the cannula is attached to a plastic tubing of the same size which runs to a graduate. A previously applied clamp on the proximal artery is released for one minute so that a one-minute flow of blood is spilled into the graduate. A point is made on the midline of the excursions on the record which corresponds to the milliliters of blood

measured. This is one point on the calibration curve. By constricting the end of the plastic tubing, lower rates of flow may be measured until a number of desired points on the flow curve may be calibrated. A curve of best fit may be drawn transecting the points and may be extrapolated to the base line. This point on the base line, or "true base" will not fall on zero, but upon an electromotive force reading which represents the "A" voltage previously described. These readings may be made identical, by means of the potentiometer adjustment. Fig. 5 shows typical calibration curves by use of two amplifications. In this calibration graph, the "A" voltage has been subtracted and it may be seen that the curves are, for all practical purposes, linear.

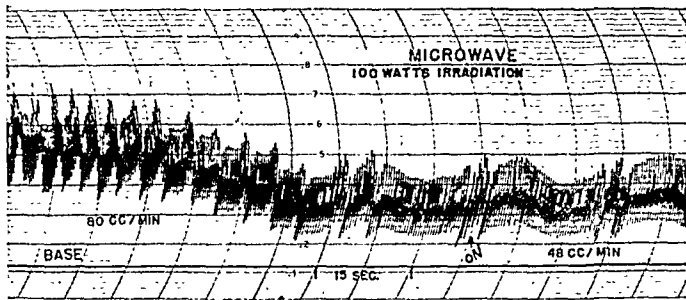


Fig. 6.—Sample record made with the new electromagnetic flow meter. The record reads from right to left.

Once it is calibrated, a cannula does not require recalibration unless it develops a fracture or similar defect. However, because of such possibilities, frequent recalibration may be advisable. The typical observation with a fractured cannula is an enormous base voltage which ordinarily can be detected readily.

#### RESULTS

Fig. 6 shows a typical blood flow record which reads from right to left. This sample record shows the results of 12 cm. microwave irradiations using 100 watts output upon the limb of a dog. The wave director was placed close to the surface of the limb. In the figure, the heavily inscribed base line represents the background pickup, or "A" voltage, so that voltage induced by blood flow reads from this point to the midline of the excursions to represent mean flow. This point at the midline was arbitrarily selected when calibrating the cannula. When this distance as read on the ordinate is multiplied by the k-value of the cannula, in this case ( $2.0 \times 10^2$ ), a figure is derived which represents blood flow in cubic centimeters per minute. For example, the control flow may be calculated as follows:

$$0.24 \times 2.0 \times 10^2 = 48 \text{ c.c./min.}$$

The calibration should be experimentally established as suggested, and the "A" voltage reading may be conveniently adjusted. Since the "A" voltage will be  $90^\circ$  out of phase with the flow voltage, they cannot be added algebraically.

Fig. 6 is a reproduction of blood flow in the femoral artery. The rapid oscillations are the result of the cardiac rhythm, while the slower oscillations are due to respirations. These events are reflected in the flow record with sufficient clarity that the record may be used to count the respirations and heart rate. It may be observed that while the rate of blood flow and respiration have been increased by the irradiations, there is not noticeable increase in heart rate. This record was made at one of the intermediate speeds. A faster speed makes the excursions much easier to analyze, while a slower speed achieves a greater economy of record.

#### DISCUSSION

While it is essentially a pulsatile flow meter, this device as described here is best adapted for the measurement of mean blood flow. As previously suggested, it is possible to better adapt this meter for the measurement of pulsatile blood flow where a finer analysis of events of a short duration is desired.

The meter as described has proved exceedingly capable for the measurement of mean blood flow in both veins and arteries. It has been found to possess less than 5 per cent error in measurement under physiologic conditions and has been operated for four- to six-hour periods with negligible voltage drift. A criticism of this type of flow meter is that general anesthesia and an anticoagulant must be used because the blood vessel is cannulated. On the other hand it is simple to operate, is substantially free from subjective factors, and possesses a relatively linear calibration curve. By use of different cannulae, the range of flow measurement is from 0 to 300 c.c. per minute. This range may be expanded with simple modifications. The meter may be easily cleaned following each use by means of a detergent and distilled water. This cleaning, carefully done, is important for proper measurements.<sup>6</sup> In brief, while the development of this meter entailed certain compromises it is felt that it has proved to be a practical, accurate, and easily operated device for the measurement of blood flow under many conditions encountered in laboratory measurement.

#### SUMMARY

A new electromagnetic blood flow meter has been developed which produces a continuous permanent record of mean blood flow. This device is adaptable to measure pulsatile blood flow in the respect that it readily records the effect of the heart rhythm and respiration upon blood flow. Modifications are suggested for recording events of shorter duration. The use of this meter entails the disadvantages inherent with cannulation, but such disadvantages must be weighed against the advantages of accuracy, stability, and ease of operation. The stability and accuracy of this meter is in a large measure dependent upon careful construction of the cannula and pickup leads.

The authors gratefully acknowledge the cooperation and assistance of Dr. J. W. Clark in the development of this work.

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# EVALUATION OF A NEW CAPILLARY RESISTOMETER: THE PETECHIOMETER

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## INTRODUCTION

CAPILLARY resistance determinations were made on one hundred patients with two different types of suction apparatus and the readings compared. A modified Dalldorf resistometer was used on one forearm as previously described,<sup>1</sup> and a new resistometer, the Petechiometer,\* was applied to the other forearm.

## MATERIALS AND METHODS

Capillary resistance is the minimal amount of suction applied to the skin for one minute capable of producing one petechia or more. It is measured in centimeters of mercury negative pressure or suction. With the modified Dalldorf resistometer, readings were made at intervals of 5 cm., the amount of suction being read directly from a vacuum gauge. The Petechiometer, on the other hand, has no vacuum gauge but is adjusted to deliver 10, 20, and 30 cm. of mercury suction. The bell of the old resistometer is 1 cm. in diameter while that of the Petechiometer is 2 centimeters.

In all of the one hundred patients two readings were noted, the old instrument being used on one forearm and the Petechiometer on the other, Fig. 1. Because in some studies<sup>2, 3</sup> readings were normally slightly lower on the right arm, it was decided to reverse machines in the second group of fifty patients. The 200 capillary resistance readings may be compared in Table I.

*Estimation of Capillary Resistance With the Petechiometer.*—The plunger is grooved at three points, each yielding a different suction. The adjustable stop ring is inserted in the innermost groove. One then places water just below the antecubital space before applying the bell to the wet skin. The air is expelled by pressure of the right thumb on the end of the plunger and the bell placed on the skin. The bell is held in contact with the skin lightly, but firmly, with pressure from the index finger of the left hand upon the top of the bell. As the thumb pressure on the plunger is released quickly a spring action applies suction to the skin area. Suction is applied for one minute and then released by pushing inward on the plunger. After removing the Petechiometer, thirty seconds are allowed before looking for petechiae. If no petechiae are found, the test is repeated using each of the stop-ring settings, i.e., the innermost yielding 10 cm. mercury suction, the middle setting 20 cm. mercury suction, and the outermost slot setting 30 cm. mercury suction. The determinations are not made closer together than the diameter of the suction bell.

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\*The Petechiometer is the registered trade mark owned by the Rexall Drug Company. The Petechiometer consists of a small suction pump with a plunger and a clear plastic Bier bell, or suction cup, 2 cm. in internal diameter, with a magnifying glass blown into the upper surface of the cup to facilitate in situ reading of the petechiae developed.

The number of petechiae produced by the Petechiometer was noted. In the final comparison of results, this procedure proved valuable, for it permitted interpolation to intervals of 5 cm. when excessive numbers of petechiae were produced at the 10, 20, or 30 cm. readings. For example, Patient 29 showed a capillary resistance of 10 cm on the Petechiometer, but had forty petechiae.

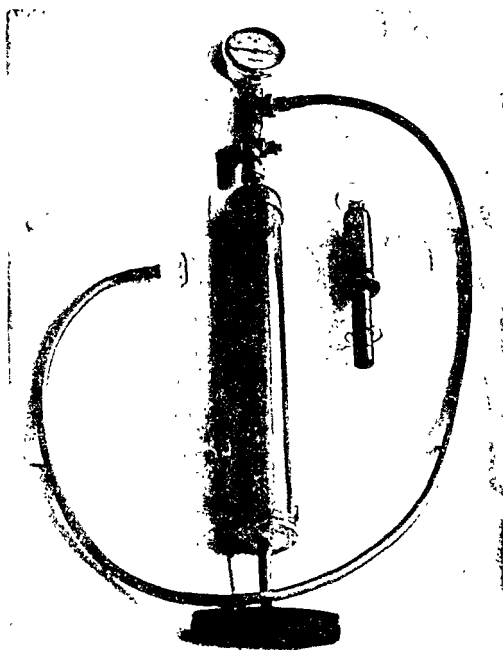


Fig. 1—Comparison between old and new resistometers

Obviously, because of the many petechiae produced, the true capillary resistance was actually less than 10 centimeters. Using the old resistometer, the capillary resistance in this patient was 5 centimeters. When comparisons were made with the two machines, it was found that when six or more petechiae were produced by the Petechiometer, one could in most instances subtract 5 cm. from the capillary resistance shown by this new instrument and approximate the reading given by the old machine.

TABLE I. CAPILLARY RESISTANCE READINGS (CENTIMETERS OF MERCURY SUCTION) IN 100 PATIENTS WITH NEW AND OLD RESISTOMETERS, USING RIGHT AND LEFT FOREARMS

	NEW RIGHT			OLD LEFT		NEW RIGHT			OLD LEFT		NEW LEFT			OLD RIGHT		NEW LEFT			OLD RIGHT
	*	P	†			*	P	†			*	P	†			*	P	†	
1.			20	15	26.			10	10	51.			30	15	76.			30	30
2.	10	8	5	5	27.			20	10	52.			10	10	77.	20	8	15	15
3.			10	5	28.	10	12	5	5	53.			20	20	78.	10	8	5	5
4.			20	15	29.	10	40	5	5	54.	10	20	5	10	79.	10	7	5	5
5.			10	10	30.			20	25	55.			10	10	80.	10	8	5	15
6.			20	15	31.			10	10	56.			10	15	81.			10	10
7.			20	15	32.			30	15	57.			10	15	82.			10	15
8.	20	8	15	20	33.			10	10	58.			20	20	83.	10	7	5	10
9.			20	15	34.			20	25	59.			10	15	84.			20	15
10.			20	25	35.			20	15	60.			10	5	85.			10	10
11.			10	15	36.			20	15	61.			10	5	86.			10	10
12.			20	20	37.			20	15	62.			10	15	87.			20	10
13.			10	10	38.	10	7	5	10	63.			20	15	88.	10	6	5	5
14.			20	25	39.	20	6	15	20	64.			20	15	89.	10	8	5	10
15.			20	20	40.			10	15	65.			10	10	90.			10	10
16.			20	15	41.	30	14	25	25	66.			10	10	91.			15	20
17.			20	15	42.			20	25	67.	20	8	15	15	92.			10	10
18.	20	8	15	30	43.	20	8	15	15	68.	10	6	5	5	93.			10	10
19.			30	25	44.			10	10	69.			20	25	94.			10	10
20.	20	12	15	25	45.	20	8	15	15	70.	20	8	15	15	95.			10	15
21.			10	10	46.			10	20	71.	20	8	15	15	96.			30	20
22.			30	35	47.			20	30	72.			20	25	97.			10	15
23.			20	25	48.			10	10	73.	20	8	15	15	98.	20	16	15	2
24.			10	15	49.	10	6	5	5	74.			20	20	99.	10	17	5	5
25.			10	10	50.			10	5	75.	20	15	15	15	100.			20	15

\*Capillary resistance before 5 cm. correction for excessive number of petechiae (six or more).

P, Number of petechiae.

†Corrected capillary resistance.

## RESULTS

By subtracting 5 cm. when six or more petechiae were noted at the capillary resistance determination with the Petechiometer, the average reading with the old instrument was 14.75 cm. and with the Petechiometer 14.25 centimeters. This similarity is not so constant in individual readings. Only 43 per cent showed no difference between old and new resistometers and 90 per cent did not vary more than 5 centimeters. Of the ten patients who differed by more than 5 cm. between arms, seven patients showed a 10 cm. difference and three patients a 15 cm. difference. One may expect, however, to find similar variations between arms even when using the same resistometer.<sup>2, 3</sup>

## DISCUSSION

A simple capillary resistometer to determine bleeding tendencies should be welcomed by physicians. It has been noted that the lower the capillary resistance, the greater is the bleeding time.<sup>4</sup> A capillary resistance of 5 cm. means marked fragility. Capillary fragility accounts for bleeding in such conditions as purpura and rheumatic fever and in hypertensive patients.<sup>3, 5, 6</sup>

Without a suction apparatus for determining capillary resistance, the clinician may resort to positive pressure methods, as by use of the sphygmomanometer.<sup>7, 8</sup> The disadvantages of such methods are listed by Tey<sup>9</sup> as follows: (1) determination is time consuming; (2) test cannot be repeated for days;

(3) there is no gradation in the same subject; (4) results cannot be read with accuracy; (5) positive pressure methods are unsuited for pharmacologic investigation, where several determinations must be made within a few minutes since only two body surfaces (arms) are available.

On the other hand, use of the Petechiometer is simple and painless. Determination of capillary resistance requires less than five minutes. The instrument is small and can be carried in one's bag.

#### CONCLUSIONS

Capillary resistance determinations were made on one hundred patients, using a modified Dalldorf resistometer with vacuum gauge on one forearm and a new instrument, the Petechiometer, on the other.

Although readings on both forearms differed in individual subjects, these differences were usually slight. Only 10 per cent of patients showed readings which differed more than 5 cm. between arms. Minor differences are normally noted between arms. These differences tended to balance in the final compilation, for the average capillary resistance with the old machine was 14.75 cm. and with the Petechiometer 14.25 centimeters. The Petechiometer thus appears to be accurate.

The Petechiometer has the following advantages over the older resistometer: it is on the market; it is simple to use; it is compact and small.

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# A SIMPLIFIED VENOUS OCCLUSION METHOD OF DIGIT BLOOD FLOW ESTIMATION USING THE BURCH-WINSOR PLETHYSMOGRAPH

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**D**IGIT blood flow studies based on the venous occlusion principle have been greatly simplified by the development of a clinical type of transmission plethysmograph by Burch.<sup>1</sup> At the present time venous occlusion blood flow determinations are being carried out as a part of the regular digit plethysmographic study on both fingers and toes in our laboratory. The basic details of this type of study have been recorded by Wilkins,<sup>2</sup> Goetz,<sup>3</sup> Abramson,<sup>4</sup> and others.<sup>6</sup> Our purpose in reporting this method is to emphasize the ease with which venous congestion methods may be adapted to the Burch-Winsor plethysmograph. Measurements with this type of apparatus depend upon volume changes in the part under study, the variables being amount and rate of volume change. For the most part an increase in the volume of a trapped part is dependent upon either increased arterial inflow or decreased venous outflow or both. If one occludes the venous outflow without disturbing the arterial inflow the volume change resulting represents an indirect estimate of the arterial inflow. The inherent limitations of this type of determination have been adequately discussed by Abramson and by others.<sup>2, 3, 4, 6</sup> We are aware of these limitations, but with increasing experience we are coming to feel that digit flow determinations are worth-while adjuncts to digit plethysmography, particularly in attempting to decide about the presence or absence of vasomotor activity in the digital blood supply.

The problem of applying an occluding pressure to the base of the digit might be mentioned first. Our first attempt was a brass-backed latex ring. This was a satisfactory method for fingers but in the case of toes a ring large enough to pass over the end of the toe was frequently too large to satisfactorily compress the toe at its base. The brass-backed latex cuffs were discarded in favor of Abramson's adhesive tape-latex tube method. In making the latex tubing for the occluding cuffs a glass rod 6 mm. in diameter which has been dusted with talcum powder is dipped into a container of prevulcanized liquid latex two or three times. After several hours of drying, the surface of the rubber on the glass rod is dusted with talcum powder and the rubber is rolled off the glass. The tubing thus obtained is thin enough to avoid bulk and strong enough to withstand the occluding pressures. The latex tubing is attached to a small catheter or plasma tubing with a fine silk binding re-enforced with a turn of adhesive tape. The latex tubing is then backed with ordinary adhesive tape.

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The end is turned under and covered with a tab of adhesive. The completed cuff is 8 to 10 cm. in length and approximately 1 cm. wide.\* These cuffs, if well made and free of bubbles, will survive a week or more of active use. In applying the cuff to the digit, the cuff is wrapped around the base of the digit and a short piece of adhesive is used to secure the cuff to itself to prevent slipping. (Fig. 1, *A* and *B*.)

The method of cuff inflation used is a simple pressure bottle arrangement similar to that of Goetz.<sup>1</sup> The essentials of the pressure system are a 1 or 2 liter widemouthed bottle, a tightly fitting rubber stopper, a large three-way glass stopcock, some type of manometer—either aneroid or mercury, a blood pressure type of compression bulb, and suitable tubing and glass or metal connections. The illustrations (Fig. 2, *A* and *B*) are self-explanatory and give a satisfactory scheme of connections for use.

In use the occluding pressures should be well below diastolic arterial pressure and we have found 60 to 70 mm. (Hg) to be satisfactory for most digits. When air is allowed to flow into a single cuff, the others having been excluded by clamps, the bottle pressure falls 1 to 2 mm. of mercury with each inflation. This is corrected prior to each occlusion by means of the compression bulb.

To summarize the steps in the digit flow determination:

1. The digit part is measured in the displacement tube apparatus<sup>1</sup> to a volume of 5 cubic centimeters. This volume is indicated on the digit by a weak gentian stain which is employed as the "measuring fluid."

2. The extremity plastic cup is sealed in place at the proximal edge of the gentian stain with the sealing compound Kalk-Kord, previously reported.<sup>5</sup>

3. After the cup seal has been tested, the occluding cuffs are applied to the base of the digit in such a way that there is no contact with the cup or the sealing compound—and no contact between the tubing from the cup and cuff. Trial cuff inflations are then carried out to make certain the cuffs are in the best alignment to prevent unnecessary motion of the digit when the cuff pressure is introduced.

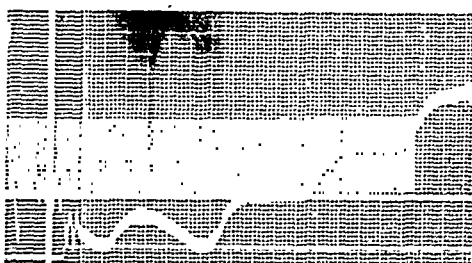
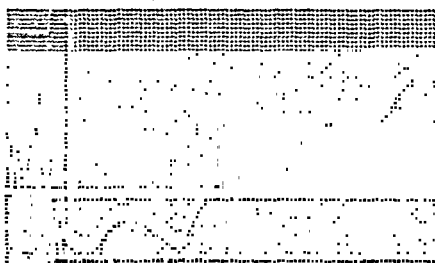
4. String sensitivity is adjusted to move 10 mm. per 10 mm.<sup>3</sup> volume change with the calibration lever. The routine plethysmographic tracings are made at slow speed, standardizing each run twice. Following the second calibration, the base line is shifted to the left side of the aperture and the camera speed shifted to "fast" just prior to the application of cuff pressure. This will require a certain amount of practice by operator and assistant. When the maximal base line shift (i.e. volume change) has occurred, the camera is shifted to slow speed and cuff pressure is released by turning the stopcock to the "exhaust" position. The base line is adjusted to the left side of the aperture, if necessary, following which the venous occlusion test is repeated. The camera is then stopped and the next digit is prepared for recording. With practice, the volume pulses and two venous occlusion runs can be recorded on about 12 inches of paper for each digit.

\*The latex which we have found satisfactory for this use is a formula designated No GL-15c, furnished by the General Latex and Chemical Corp., 566 Main Street, Cambridge, Mass.

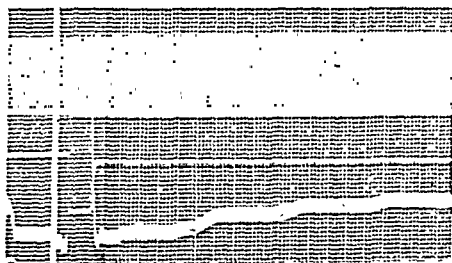
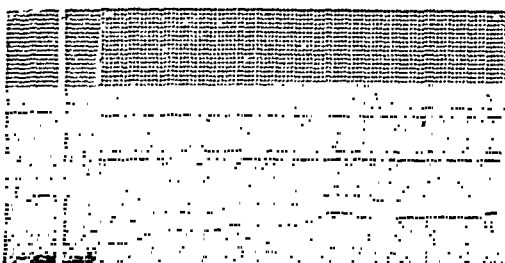
# Venous Occlusion Test

Name: A.H. ♀  
Date: 13 June '49

Digit: Left 1st Toe



Warm Room  
Temp.: 83°F.



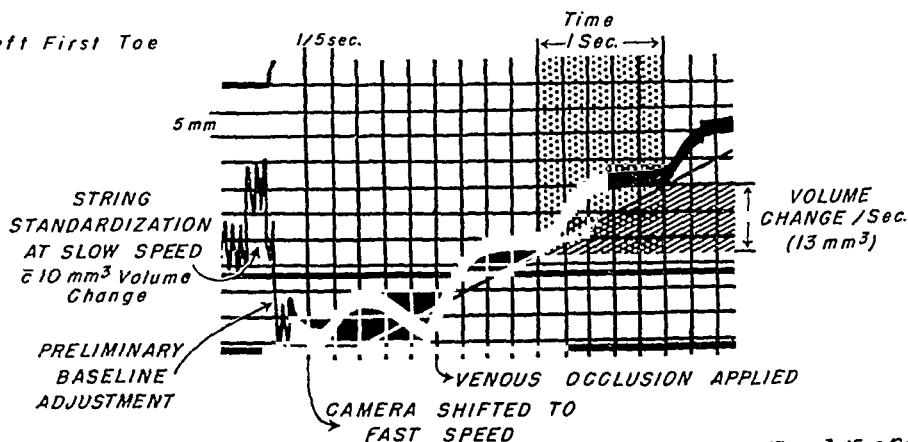
Cold Room  
Temp.: 68°F.

A.

## ESTIMATION OF DIGIT FLOW (mm<sup>3</sup>) / 5 cc. part / sec.

AH ♀ 6/13/49

Left First Toe



DIGIT FLOW = 13 mm<sup>3</sup> / 5cc./Sec.

B.

Fig. 3.

5. After the tracing has been developed, the digit flow can be readily determined by measuring the amount of volume change per unit of time (Fig. 3, *B*). We have arbitrarily used millimeters<sup>3</sup> per second per 5 c.c. of part. In drawing the tangent slope line it is desirable to avoid using the first pulse excursion if possible since positive or negative artifacts are common. In rapid flows this may not be possible because of the speed with which the string shadow crosses the entire width of the paper, in which case the slope line should be placed along the most representative portion of the curve. In connection with measuring the various string deflections on the tracing, a correction formula has been used to compensate for "high" or "low" calibration errors. The correction ratio which we employ uses the following factors:

$$\frac{\text{Measured volume deflection} : \text{True volume deflection (X)}}{\text{Measured calibration deflection} : 10}$$

Thus if the measured volume deflection were 14 mm. and the standardization deflection were found to be 8 mm. instead of 10 mm. the measured deflection would be low and by the correction formula the true volume deflection would be:

$$\begin{aligned} 14 : X &= 8 : 10 \\ SX &= 140 \\ X &= 17.50 \text{ mm./5 c.c. of part (corrected deflection).} \end{aligned}$$

Although this formula does not take into consideration all the variable factors which might cause minor errors, such as variation in volume of the trapped part, it is probably worth while in measuring volume pulses and blood flow where the standardization error is greater than 1 millimeter.

The illustrations demonstrate the essential details of this method of blood flow estimation in digits. The plethysmograms presented are tracings from normal individuals who have no evidence of peripheral vascular disease. The "warm room" flows have been recorded after one hour in an ambient temperature of 83° to 84° F. and the "cold room" flows after one hour in an ambient temperature of 68° to 69° F. (Fig. 3, *A* and *B*.)

The application of this type of digit blood flow determination to normal subjects and before and after limb sympathectomy will be presented in subsequent communications.

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# PROCEEDINGS OF THE CENTRAL SOCIETY FOR CLINICAL RESEARCH

Twenty-Second Annual Meeting

Chicago, Ill., Nov. 4 and 5, 1949

## ABSTRACTS—Concluded

### 61. EXPERIMENTAL PRODUCTION OF MEGALOBlastic ANEMIA; AN INTERRELATIONSHIP BETWEEN ASCORBIC ACID AND PTEROYLGLUTAMIC ACID

CHARLES D. MAY, M.D., E. N. NELSON, M.D. (BY INVITATION), AND  
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This report describes a means of producing megaloblastic and macronormoblastic anemia in monkeys using deficient diets alone.

The diets employed were basically dried cow's milk reconstituted with water and adjusted with lactose and vegetable oils and supplemented with crystalline vitamins to provide liquid diets of the following approximate composition:

	PER 100 ML.
Protein	1.6 Gm.
Carbohydrate	7.2 Gm.
Fat	3.3 Gm.
Calories	67
Vitamin A	800 I. U.
Vitamin D	50 I. U.
Thiamin	.07 mg.
Riboflavin	.1 mg.
Nicotinic acid	.5 mg.

Any other vitamins present were those contained in the milk. Each experimental animal was given .137 mg. Fe per kilogram in the form of iron ascorbate intravenously at the start of the experiment followed by several weeks of oral therapy with 4 c.c. of ferrous gluconate daily.

In five control periods monkeys fed this type of diet ad libitum and 50 mg. ascorbic acid daily for five or more months remained in good health and did not develop anemia or changes in the bone marrow.

When the experimental diet was fed to eleven monkeys without the addition of ascorbic acid, a typical sequence of events occurred. For three to four months they remained in apparent good health. At about this time the first symptoms of scurvy appeared. They remained essentially in this state for the next three to four weeks, exhibiting only the symptoms of scurvy, without developing anemia or definitive changes in the marrow. Then a precipitous deterioration began. Pronounced anorexia and listlessness ushered in this phase. Diarrhea commenced invariably, the stools being loose to mushy, bulky, and foul but without pus or blood. With one exception a histamine refractory gastric achlorhydria developed. The fur lost its smoothness and luster and was shed in large amounts. The peridontal gingivae became ulcerated and necrotic. No changes were seen in the tongue. No infections were observed in the animals at any time. Anemia and neutropenia developed rapidly without evidence of additional hemorrhage. The anemia was usually of a normocytic or macrocytic type.

The most critical criteria were applied in designating the types of cells seen in the marrow. Three monkeys developed megaloblastic marrows very similar to, if not identical with, those seen in megaloblastic anemia in human

beings. The marrows in the other eight animals became macronormoblastic, a stage through which the megaloblastic marrows passed. The alterations in the granulocytes in each instance were striking, showing hypersegmentation and large early forms with premature lobulation such as are seen in the marrow in pernicious anemia. It was the impression that all the marrows might have progressed to a frankly megaloblastic picture if the precarious condition of the animals had not made it seem necessary to treat them in order to test the effects of various agents.

A systematic trial of vitamin B<sub>12</sub>, PGA, and ascorbic acid revealed the following: (1) Ascorbic acid given relatively early in the disease gradually restored the animal to health and the marrow and blood became normal. (2) If ascorbic acid was not given until an advanced stage of the disease, it did not restore the marrow to normal or prevent the death of the animal. (3) Vitamin B<sub>12</sub> alone, given intramuscularly, had no effect on the marrow within forty-eight hours. (4) PGA, given orally or intramuscularly as the free acid or the triglutamate, but without ascorbic acid, promptly stimulated normal hematopoiesis in the marrow and relieved all the symptoms not attributable to scurvy. When ascorbic acid was added, normal health was restored even though the experimental diets were continued.

Increasing the protein in the diet to 3.3 Gm. per 100 c.c. by adding calcium caseinate did not prevent the development of the characteristic anemia.

Thus it would appear that megaloblastic anemia developed as a result of PGA deficiency somehow induced by a chronic deficiency of ascorbic acid.

## 62. CERTAIN EFFECTS OF CHEMOTHERAPY ON THE FECAL AEROBIC AND ANAEROBIC BACTERIA OF PATIENTS WITH CHRONIC ULCERATIVE COLITIS

HOMER C. MARSHALL, M.D. (BY INVITATION), WALTER L. PALMER, M.D.,  
AND JOSEPH B. KIRSNER, M.D., CHICAGO, ILL.

The role of the fecal bacteria in the pathogenesis and course of chronic nonspecific ulcerative colitis is not clear. A method of simplifying the bacterial flora of the feces and reducing the number of bacteria in the bowel would provide useful information concerning this problem. Accordingly, qualitative and quantitative studies of the aerobic and anaerobic bacteria were made before and after treatment with absorbable and nonabsorbable sulfonamides, penicillin administered orally and parenterally, and the oral administration of streptomycin, aureomycin, and chloromycetin. Control studies consisted in observations intended to determine the error of the method and to ascertain the spontaneous day-to-day variation. The drugs were administered for relatively long periods of time; bacteriologic studies were carried out at frequent intervals in order to determine the initial and late effects of chemotherapy. All of the drugs, under certain conditions, are capable of altering the aerobic flora; some also may modify the anaerobic flora of the feces, the degree and duration of effect varying considerably. For instance, sulfonamides alter the aerobic flora, when initially used, for periods as long as six weeks. Streptomycin, on the other hand, exerts a marked quantitative effect, which is maintained, however, only for several days. Chloromycetin and aureomycin exert an intermediate effect: the *B. coli* disappear and the total bacterial count initially falls and then rises, with the reappearance of *B. coli* as a predominant organism. In addition, after the initial depression of bacterial counts had terminated, the counts tended to rise above the control levels and to remain high, decreasing to control levels only after the drugs were discontinued.

All of the patients improved when a basic regimen was instituted which provided a diet which contained calories equivalent to twice the calculated basal requirement;  $2\frac{1}{2}$  Gm. of protein per kilogram of body weight; 30 per cent of the calories as fat and the remainder as carbohydrate. The patients' clinical improvement was accelerated by the addition, to this regimen, of daily intravenous infusions of a mixed amino acid solution which was sodium free.

Restriction of the sodium intake below 1.0 Gm. per day was a valuable adjunct in combating fluid retention. The patients with edema and ascites showed an increased urinary output and the edema and accumulation of ascites diminished when salt intake was restricted.

No clinical benefits were observed in the patients when the basic regimen was supplemented with cystine, methionine, choline, B complex vitamins, or parenteral liver.

The infusion of large amounts of concentrated human serum albumin (salt poor) was impractical as a method of protein supplementation, even though a transient rise in serum albumin concentration and a marked increase in positive nitrogen balance attended its administration.

On the regimen described (diet, salt restriction, and supplementation with amino acid infusions) all of the patients showed increased weight gains without fluid retention; those with edema and ascites gradually showed reduction and disappearance of these manifestations of liver disease. All patients showed a striking capacity for the retention of tremendous amounts of nitrogen, probably due to replacement of fluid in the tissues by newly synthesized muscle protein.

One patient with advanced cirrhosis and recurrent ascites was observed over a period of two years while subsisting on seven different regimens. At the peak of the patient's illness, paracenteses were necessary at ten to twenty-day intervals and 16 to 19 liters of fluid were removed at a time. Albumin therapy produced no alteration in the rate of accumulation. A low salt regimen reduced the frequency of paracenteses to about thirty-day intervals. Finally, with the previously described regimen, the patient became ascites free and has remained so for 7 months. He has maintained his body weight gains and has returned to a normal active existence.

Of seven liver function tests, serum cholinesterase activity was the most reliable test of liver function in that the cholinesterase levels most accurately correlated with the patient's clinical condition.

## 67. QUANTITATIVE STUDIES OF VIBRATORY PERCEPTION IN DIABETIC AND NONDIABETIC SUBJECTS

I. ARTHUR MIRSKY, M.D., PERRY FUTTERMAN, M.D. (BY INVITATION), AND ROBERT H. BROH-KAHN, M.D., CINCINNATI, OHIO

Quantitative measurements of the threshold of vibratory perception were made in 102 diabetic patients and in 136 nondiabetic, nonobese, nonhypertensive subjects. Both groups of patients were chosen at random from an ambulatory population and were from 5 to 79 years of age.

No correlation was found to exist between the threshold of vibratory perception and the side of the body tested or the sex of the subject. A significant increase in the threshold was found in the diabetic group of subjects. This increase was not related to either the known duration of diabetes mellitus or the severity of the metabolic derangement as estimated by the patients' insulin requirements. Diabetic subjects with symptoms or signs of neuropathy tended to have a greater incidence and greater degree of vibratory

impairment than those without such signs or symptoms. Similar measurements in nondiabetic groups of obese and hypertensive patients revealed no differences in their thresholds from those of the control group.

Both the diabetic and nondiabetic groups of subjects showed an increasing threshold of vibratory perception with increasing age. This increase was more marked in the toes than in the fingers and was greater in the diabetic than in the nondiabetic subjects. The data revealed a more rapid increase in vibratory threshold among the diabetic subjects during the earlier decades; during the later years of life, the threshold of the nondiabetic subjects rapidly approached the level of the diabetic subject.

This study provides evidence for the concept that the process of aging is more rapid, or of greater initial intensity, in the patient with diabetes mellitus than in the nondiabetic subject.

## 68. MECHANISM OF HYPERTHERMIA NOT DUE TO INFECTION

MAX M. MONTGOMERY, M.D. (BY INVITATION), FORD K. HICK, M.D., AND  
ROBERT WOOD KEETON, M.D., CHICAGO, ILL.

Fever usually indicates infection but also occurs when an individual cannot eliminate heat produced by his body metabolism, a principle utilized in fever production by the various "cabinets."

In the past few years a number of patients have been observed with fever not easily ascribed to infection. Records of nine such patients permitted study of the rectal temperature, state of the skin surface, pulse rate and rhythm, blood pressure, and environmental conditions. Six developed fever ranging from 104 to 107.8° F. during hot humid weather, and three developed fever ranging from 102.8 to 106° F. during moderate weather. Six were postoperative and 3 were medical patients.

Failure of transport of heat from the interior to the body surface with consequent heat retention was indicated in five of the postoperative and two of the medical patients by a cold skin and a high rectal temperature. In each case a circulatory factor was present, auricular fibrillation in two, excessive tachycardia in two, severe hemorrhage in one, shock following myocardial infarction in one, and circulatory collapse in acute liver necrosis in one.

Failure to eliminate heat from a hot skin was present in one patient with ichthyosis who was virtually unable to sweat and in whom anesthesia, surgery, and excessive bed covering combined with very hot weather to produce a rectal temperature of 105° F. An overdose of barbiturate led to hyperthermia of 104.6° F. in one patient found unconscious in a hot humid room. Dehydration, pulmonary edema, and anoxia plus a disturbance of the heat regulatory mechanism were contributory factors.

In each patient several factors were operative, but the common factor was the inability to lose heat as rapidly as it was produced, usually due to inadequate circulation. A hot humid environment was often the precipitating factor since this interfered with heat loss. The load on the cardiovascular system was increased by heat retention and attendant tachycardia. This led to excessive tachycardia or auricular fibrillation with less efficient circulation and further heat retention.

From the study of these patients with severe hyperthermia one is led to consider the factor of heat retention in fevers of lesser degree such as occur in congestive heart failure, shock, myocardial infarction, etc. Measures aimed at facilitating heat loss at the inception of fever may prevent the serious effects of hyperthermia.



## 69. THE EFFECT OF HIGH VAGUS SECTION UPON THE CLINICAL PHYSIOLOGY OF THE BRONCHI

DOUGLAS R. MORTON, M.D. (BY INVITATION), KARL P. KLASSEN, M.D. (BY INVITATION), AND GEORGE M. CURTIS, M.D., COLUMBUS, OHIO

Other than the occurrence of substernal pain in patients having acute bronchitis, little is known concerning pain of bronchial origin. Consequently, an evaluation of the quality as well as the referral of pain of bronchial origin was made in patients by direct stimulation of the bronchial mucosa, using an electrode introduced through a bronchoscope. Following recovery from the bronchoscopy, the patients described the sensation as a moderately intense aching pain, which was felt in the homolateral anterior chest or the homolateral anterior cervical region. In a series of twenty-five patients thus investigated, the referral of pain was consistent and symmetrical.

Patients found to have inoperable bronchogenic carcinoma at exploratory thoracotomy afforded an opportunity to evaluate the effect of vagotomy on the clinical physiology of the bronchi. Accordingly, in these patients the vagus nerve was transected above the pulmonary plexus, yet below the origin of the recurrent laryngeal nerve, with the following results:

(1) The *cough reflex* arising from the homolateral bronchial tree was abolished in all instances. (2) In the majority of cases *pain* of bronchial origin was abolished on the homolateral side. In the remaining few, there was referral of pain to the contralateral anterior cervical region. (3) No effect on the physiologic respiratory change in *bronchial caliber* was noted, nor was any effect observed on *bronchial motility*. (4) On bronchoscopic observation no subsequent changes were noted in the amount nor in the consistency of *bronchial secretions*; likewise, postbronchography roentgenograms revealed no impairment of Lipiodol clearance of the tracheobronchial tree. (5) In a limited number of cases, electrocardiographic tracings were unaffected by compression of the vagus nerve or its transection or by electric stimulation of either the proximal or distal ends of the transected vagus.

## 70. PYRIDOXINE DEFICIENCY IN HUMAN BEINGS INDUCED WITH DESOXYPYRIDOXINE

JOHN F. MUELLER, M.D. (BY INVITATION), AND RICHARD W. VILTER, M.D., CINCINNATI, OHIO

Since 1934, when György announced the existence of a new vitamin for the rat, which when synthesized was called pyridoxine, numerous reports have appeared clearly defining deficiency states in various laboratory animals. These have included seborrhea-like dermatitis, muscular weakness, decrease in lymphoid tissues, and decrease in circulating antibodies in the rat, hypochromic anemia in the dog, anemia and central nervous system lesions in swine, and lymphocytopenia and absolute increase in neutrophils in the monkey. There has never been a clear-cut demonstration that pyridoxine is essential to human beings and a pyridoxine deficiency state in man is unknown.

With the recent advent of metabolite inhibitors, a new tool has been placed in the hand of the investigator to study deficiency states. Such antagonistic activity toward pyridoxine has been demonstrated for desoxypyridoxine in the chick. During pilot experiments to determine the toxicity of desoxypyridoxine in man, we observed a deficiency syndrome precipitated

by a vitamin B complex-poor diet and desoxypyridoxine which responded specifically to pyridoxine. These early experiments have been extended and are the basis of this report.

Eight patients suffering from various chronic illnesses were given 60-150 mg. of desoxypyridoxine intramuscularly daily while they were maintained on a diet low in vitamins of the B complex. Hematologic studies were done before and at biweekly intervals after the institution of the experimental regime. Urine assays for pyridoxic acid, thiamin, riboflavin, and N<sup>1</sup>-methylnicotinamide were done at weekly intervals in most instances. When the deficiency state developed, the patient was placed on a vitamin B complex mixture devoid of pyridoxine and later on pyridoxine alone. The desoxypyridoxine and diet were continued throughout the entire experimental period.

Seborrhea-like skin lesions developed about the eyes, nose, and mouth within two to three weeks in seven of the eight patients. In three of the seven the lesions were quite severe. Half of the patients developed erosions in and around the mouth resembling cheilosis of riboflavin deficiency and glossitis resembling morphologically that seen in niacin deficiency. One patient developed severe systemic symptoms. A mild but definite lymphocytopenia appeared in seven of the eight patients. No anemia occurred. These manifestations were unchanged when the vitamin B complex mixture devoid of pyridoxine was given but responded in forty-eight to seventy-two hours to the parenteral administration of 100 to 200 mg. of pyridoxine.

The authors feel that these lesions constitute at least one part of the human syndrome of acute pyridoxine deprivation.

## 71. PANCREATIC DYSFUNCTION AND LIVER DISEASE

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AND VINCENTE MORAGUES, M.D. (By INVITATION), ST. LOUIS, MO.

Advances have been made in the knowledge of the etiology and pathogenesis of liver and pancreatic diseases. Recent animal studies by Chaikoff, Best, Gillman, Ivy, and others have shown that fatty deposition in the liver or fibrosis may follow inflammatory changes in the pancreas, pancreatic duct ligation, and pancreatectomy in dogs maintained on a diet of lean meat and insulin. The hepatic changes could be prevented by the feeding of pancreatic tissue and lipotrophic agents.

Experimentally there appears to be sufficient evidence to indicate that disturbance in pancreatic function may lead to serious liver disease.

Hepatic changes have been demonstrated to accompany certain pancreatic diseases such as diabetes mellitus and acute pancreatitis. However, pancreatic dysfunction as a cause of serious permanent liver damage has largely been overlooked.

The use of frequent serum diastase determinations and the prostigmine diastase test has increased the accuracy and incidence in the diagnosis of pancreatic dysfunction and has enabled us to detect abnormalities of the pancreas when diseases of other organs in the upper abdomen may be also present.

We wish to report the case histories and laboratory data of five patients who had pancreatic disease as shown by the clinical history, elevated random diastase determinations, and abnormal prostigmine diastase tests and who also had hepatic disease. These diagnoses were confirmed by surgical exploration and biopsy.

The pathology in the liver ranged from periportal round-cell infiltration, similar to that seen in cholangiolytic hepatitis, to the marked fibrosis of advanced hepatic cirrhosis.

In none of the cases could the usual etiological agents of liver disease such as toxins, infection, or malnutrition be elicited. The elevated serum diastase values which occurred at intervals in these patients as well as the abnormal prostigmine diastase test are at variance with the low serum diastase values usually found in patients with damaged livers. These findings and the experimental work of Chaikoff and others, which indicate that removal or damage of the pancreas in dogs and rats will result in fatty infiltration of the liver, as well as inflammatory changes and various types of fibrosis, lead us to suspect that pancreatic dysfunction may at times play an important etiological role in the pathogenesis of liver disease.

## 72. CHANGES OBSERVED FOLLOWING THE EXPERIMENTAL INFUSION OF THE DIURETIC SODIUM SULFATE

L. A. NALEFSKI, M.D., CHICAGO, ILL.

(INTRODUCED BY N. C. GILBERT, M.D.)

The diuretic ability of sodium sulfate was established some sixty years ago in experimental animals. It was first administered parenterally to human subjects in 1934. No adverse effects were reported in normal subjects receiving moderate doses, and when given to patients with diminished urine outputs, it appeared to be nontoxic. Maitland encouraged its use for anuria resulting from crush injuries during the "blitz" in England, but it was not widely adopted. Recently the Council on Pharmacy and Chemistry of the American Medical Association granted its approval for the parenteral use of sodium sulfate as a diuretic in the treatment of oliguria or anuria secondary to the crush syndrome, burns, transfusion reactions, toxic hemolysis, and obstructive lesions of the genitourinary tract.

The author observed the use of this diuretic in four patients. In two with cardiac edema, one responded well and the other expired suddenly several hours after 1 liter of isotonic sodium sulfate was administered. Two patients with anuria resulting from hemolytic reactions consequent to receiving improperly typed blood expired without diuresing. Unusually high blood sulfate levels were found following the use of sodium sulfate.

The foregoing clinical observations prompted further experimental study of this drug. Healthy female dogs were anesthetized, after which they were infused with isotonic sodium sulfate. A control group of animals was studied using isotonic sodium chloride. The infusions were maintained for a period of five hours. At hourly intervals arterial blood samples were obtained and subjected to a complete electrolyte and acid-base study. Urine was collected quantitatively during the same intervals and studied similarly. Electrocardiographic tracings were made initially and at the end of each hour. At the end of the five-hour infusion the animals were sacrificed and tissue biopsies obtained.

It was found that a true acidosis developed following sodium sulfate infusion and that this acidosis could be mitigated by the proper use of buffers in the infusion media. Histologic changes were present in the kidneys of those animals infused with sodium sulfate and absent in the control group. Electrocardiographic changes were also demonstrated during the sodium sulfate infusion.

It is concluded that the indiscriminate use of sodium sulfate as a diuretic in cases of anuria is dangerous. By utilizing a buffered solution with the sodium sulfate, it is thought that some of this danger may be eliminated.

### 73. CARDIOVASCULAR CHANGES FOLLOWING THE EXPERIMENTAL ADMINISTRATION OF BARIUM CHLORIDE

L. A. NALEFSKI, M.D. (BY INVITATION), N. C. GILBERT, M.D., AND  
G. K. FENN, M.D., CHICAGO, ILL.

During recent experiments with various heparin preparations it was found that the sodium salts of heparin increased the coronary flow volume, while the barium salts of heparin did not show this effect. In earlier work in coronary flow studies we gained the impression that the soluble barium salts acted as a coronary constricting agent. We therefore decided to study the action of this drug on the cardiovascular system in detail.

Healthy mongrel dogs, ranging in weight from 10 to 12 kilograms, were given barium chloride orally and parenterally in a conscious state. Electrocardiographic and plethysmographic tracings were made. Blood pressures were recorded. The effect on coronary flow was determined in anesthetized animals by cannulating the coronary sinus and also by studies on the empty beating heart.

Barium chloride administration was followed (a) by electrocardiographic changes such as seen in coronary insufficiency and (b) by a variety of arrhythmias. A hypertensive effect was produced with a slowing of the heart rate. The plethysmographic tracings indicated a generalized vasoconstrictor effect. Coronary flow was markedly decreased in the experiments conducted.

There appeared to be a difference in tolerance of the drug in various animals. Some would suddenly expire with agonizing cries, and electrocardiographic tracings in these animals indicated that death was caused by coronary insufficiency. Autopsies made immediately after death showed grossly areas which were similar in appearance to those found immediately following ligation of a coronary artery. All of these toxic effects of barium chloride could be delayed or prevented by treating the animal previously or during the experiment with aminophyllin or papaverine.

It has been shown by others that barium chloride is precipitated within a matter of several minutes by the sulfate ions normally present in the blood stream. Therefore, it was difficult to explain the prolonged effects that barium chloride administration sometimes exhibits. Because of this paradox, colloidal barium sulfate preparations were made and given parenterally. Similar electrocardiographic changes were obtained with this molecular preparation as with the ionized salts.

From these experiments we conclude that the clinical use of barium chloride in the Stokes-Adams syndrome, or for any other purpose, is dangerous and unpredictable.

### 74. KIDNEY EXCRETION DURING AND AFTER HEMOGLOBINEMIA

A METHOD FOR PRODUCTION OF HEMOGLOBINEMIA BY HIGH SONIC VIBRATION

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Our goal in this work was to obtain an experimental animal (dog) with complete suppression of kidney excretion due to hemoglobinemia, the animal to remain anuric and die in uremia from five to ten days after the onset of hemoglobinemia. Acute and chronic experiments were done on large, healthy, male dogs.

Acute experiments were performed using the following methods for producing hemolysis: thermal trauma, intravenous injection of distilled water, intravenous injection of hemoglobin solution, intravenous injection of hemolytic agents, heating and cooling blood, and, finally, use of high frequency sonic vibrations. The latter method proved to be the most useful, and it was employed throughout most experiments. Blood pressure, urine excretion, blood studies, and survival time were studied. For production of the sonic hemolysis, a magnetostriction oscillator was used that produced high energy by giving off vibrations in the region of high sonic frequency. The magneto-oscillating rod was placed into a glass cup with two side cannulas which were connected to blood vessels. The blood was hemolyzed as it flowed past the rod. An extremely high degree of hemoglobinemia could be obtained; the highest value was 11,125 mg. per cent of plasma hemoglobin.

In well-hydrated animals with no hemoconcentration, hemoglobinemia may produce complete suppression of kidney excretion during the period of hemolysis. Following this the animal may develop diuresis when plasma hemoglobin is below 5,000 mg. per cent; oliguria when plasma hemoglobin is between 5,000 and 8,000 mg. per cent; or anuria when plasma hemoglobin is above 8,000 mg. per cent. In an animal with moderate reduction of blood pressure, together with a progressing hemoconcentration, the production of hemoglobinemia with levels above 2,500 mg. per cent resulted in anuria.

The duration of the period in which hemolysis was produced varied between ten and sixty minutes. It is felt that, for the study of experimental anuria, rapid hemolysis must be used, in order to obtain comparable experimental animals.

## 75. CARDIAC FACTORS IN "NEUROGENIC" PULMONARY EDEMA

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Experimental evidence indicates that Starling's principles of fluid balance are applicable to the lung. Pulmonary edema follows either a diminution of blood osmotic tension or an increase in capillary hydrostatic pressure. Furthermore, increased pulmonary hydrostatic pressure, sufficient to provoke edema, results when cardiac overload is unequally thrown upon the left ventricle. It is likewise possible that left ventricular overload may be produced by "neurogenic" factors. Increased intracranial pressure or vagal stimulation are reported to cause pulmonary congestion and edema through cardiovascular responses extraneous to the lungs.

In open-chest preparations of dogs, pulmonary and systemic arterial and venous pressures were simultaneously recorded. The administration of epinephrine (0.25 to 1.0 mg. intravenously) produced the usual striking rise of arterial pressure and marked bradycardia. Cardiac dilatation and elevation of pulmonary venous and arterial tensions occurred, with congestion and edema of the lungs. Conversely, when bilateral faradic vagal stimulation was applied, the resulting bradycardia was accompanied by a fall of systemic arterial pressure; pulmonary congestion and edema did not occur. It is suggested that sufficient elevation of systemic arterial pressure, together with critical bradycardia (reduced minute volume output), from epinephrine, may overload the left ventricle if there is continued competence of the right ventricle and optimum venous return. The experiments suggest that neurogenic influences may alter the pulmonary vascular dynamics through adjustments that lead to impaired left ventricular function.

## 76. DIABETES DETECTION

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(INTRODUCED BY MAURICE HARDGROVE, M.D.)

Diabetes detection is becoming a problem of increasing importance. Early diagnosis may be the only way one can hope to modify or possibly cure the disease. This is a preliminary report on a diabetes detection survey started in September, 1948, which is still being carried on at an industrial plant in Milwaukee, Wis. To date, 180 patients have been tested, 69 female and 112 male, their ages ranging from 18 to 75 years. The patients are an unselected group who come to the medical department for routine physical examinations or come with minor ailments. No patient with a definite history of diabetes or symptoms suggestive of diabetes is included in the present report. The individual, besides having routine laboratory examinations, was given 100 Gm. of glucose on a fasting stomach and a blood sugar determination two hours after the ingestion of the glucose. The blood sugar determinations were done by the Folin-Wu method; recently the Somogyi method has been used in addition.

If the blood sugar report was 130 mg. or over, the patients were told to eat a normal diet during the next three days and warned against missing meals or fasting. After that period a glucose tolerance test was done. In those patients who fulfilled the following criteria, presence of sugar in the urine together with peak level above 170 mg. per cent (Folin-Wu) after the ingestion of glucose, a diagnosis of diabetes was made. Eleven cases fell in this group.

Five patients were diagnosed as having a prediabetic state, because these patients demonstrated diabetic glucose tolerance curves but did not have sugar in the urine. A diabetic curve is one with a peak level above 170 mg. per cent and a level above 130 mg. per cent two hours after ingestion of 100 Gm. of glucose. In these five patients the peak levels ranged from 196 to 216, and none had a return of B.S. to below 132 in two hours. Two patients were classed as potential diabetic patients. They had peak levels of 225 and 187, respectively, and two-hour levels of 108 and 114 with no sugar in the urine.

On the basis of this survey, eleven patients, or 5.1 per cent, had definite diabetes, as proved by an abnormal glucose tolerance and sugar in the urine. Five patients (2.8 per cent) had abnormal glucose tolerance curves but had no sugar in the urine, and two (1.1 per cent) were classified as potential diabetic patients. None of these patients have symptoms of diabetes. Some have had several determinations during the year and continue to show the same abnormal tolerance curves. Only seven of these patients have fasting blood sugars above 120 mg. per cent, the highest being 151 mg. per cent.

If one considers the first two groups collectively, approximately 7.9 per cent of the patients will have diabetes. It is felt that screening methods for diabetes will uncover a large number of diabetic patients, but many will still be missed unless more accurate testing is done.

## 77. INTERPRETATION OF THE RESULTS OF THE FLOCCULATION TESTS ON BASIS OF BIOPSY FINDINGS AND PROTEIN PARTITION

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The diagnostic significance of the flocculation tests in hepatobiliary diseases is not fully understood despite their established practical value. Therefore, in 187 cases with various liver diseases (226 series of determinations), the results of cephalin flocculation (CF), thymol turbidity (TT), zinc sulfate turbidity (ZST), and the recently described gamma globulin turbidity (GGT) were compared with those of chemical and electrophoretic partition of serum proteins into albumin and the various globulins and, where available, with the findings in liver biopsy specimens. This correlation indicated the following factors as influencing the results of the flocculation tests.

1. Reduction of serum albumin, mostly as result of liver cell damage. This increased all flocculation tests except the GGT.

2. Elevation of gamma globulins in hepatic disorders mostly related to proliferation of Kupffer cells and other mesenchymal cells and histologically recognized by appearance of cytoplasmic ribonucleic acid. This is possibly an expression of antibody formation or of stimulation by liver cell breakdown products. This factor tends to elevate all flocculation tests. It also explains the elevation of GGT and ZST in recovering viral hepatitis, being more marked with tendency for cirrhosis formation.

3. Depression of gamma globulin formation in severe jaundice as result of intra- or extrahepatic biliary obstruction, apparently related to excessive bile pigment imbibition of the Kupffer cells.

4. Depression of ZST but also CF and TT (despite elevation of the gamma globulins) by a humoral factor occurring in obstructive jaundice (probably of biliary nature) and occasionally in cirrhosis without jaundice (so far of unknown character).

5. A factor probably resulting from qualitative changes of serum albumin producing elevation of CF and TT, not explained by changes in the albumin/gamma globulin ratio.

6. Lack of a TT enhancing factor, possibly of lipid character but not related to total lipid or cholesterol concentration. This accounts for the often normal or only slightly increased TT in alcoholic cirrhosis.

Interplay of these factors produces diagnostic patterns of the flocculation tests in most conditions. Only in toxic hepatitis was the pattern not characteristic. The third and fourth factors account for the normal flocculation tests in extrahepatic biliary obstruction and occasionally in hepatitis with intrahepatic obstruction, both even in presence of severe liver damage. Superimposed bacterial infection in extrahepatic obstruction may raise GGT, CF, and TT without necessarily elevating ZST. Understanding of these factors and the performance of all four flocculation tests increases their diagnostic value.

## 78. A PRELIMINARY REPORT ON THE STUDY OF MYOCARDIAL INFARCTION BY AURICULAR CATHETERIZATION

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In a preliminary experience initiating a study of the dynamic effects of acute myocardial infarction, six patients were studied by the right auricular catheterization technique. All patients had definite evidence of the onset of an acute infarct both from clinical and electrocardiographic studies ten to seventeen days prior to catheterization. Cardiac rhythm was normal and congestive failure absent. This postinfarction period was selected as the period of initial study because of the theoretical hazards accompanying catheterization immediately following an acute infarct. After sufficient data and experience have been acquired in the study of this period, observation will be extended to the more immediate postinfarction period.

In nine control patients hospitalized for a similar period of time with diseases other than cardiac disorders, cardiac indices ranged from 2.8 to 3.8 liters per minute per square meter of body surface.

No difficulties or complications have been encountered during or after catheterization. Basal oxygen consumption and right auricular pressures during the procedure were within normal limits in all patients.

In three patients, cardiac output, arterial blood pressure, and heart work were within normal limits. In the remaining three patients, all of whom had low blood pressures, work was reduced approximately 30 per cent below the lower normal range of the control values.

One patient was catheterized on the tenth and again on the thirtieth postinfarction day. At the first catheterization values for output and work were low. Following the usual clinical improvement during the following three weeks, blood pressure and work rose, but cardiac output remained unchanged.

These studies are of a preliminary nature and no conclusions relative to factors leading to reduced output or maintenance of a normal output following infarction can be drawn. In the present small series of cases, there was no correlation between the apparent size of the infarct determined from the electrocardiogram and the level of cardiac output.

## 79. TWO RARE CASES OF CONGENITAL MALFORMATION OF THE HEART OF THE CYANOTIC GROUP; RIGHT HEART CATHETERIZATION AND ANGIOCARDIOGRAPHIC STUDIES

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AND N. GROSSMAN (BY INVITATION), CHICAGO, ILL.

Two cases of rare anomalies of the cyanotic group which must be differentiated from the tetralogy of Fallot are presented.

The first case is that of a girl  $5\frac{1}{2}$  years of age with symptoms of mild dyspnea and cyanosis on exertion. . . . . and angiocardiography demonstrate a complex . . . . . of (a) a single ventricle, (b) a pseudotruncus ar . . . . . hypoplasia of the right pulmonary artery, and (c) aberrant venous coronary drainage directly



into the common ventricle. Data and pressure tracings are presented. Differential diagnosis and certain hemodynamic factors, particularly those which may disturb the balance between the systemic and pulmonary circuits, are discussed.

The second case is that of a 4-year-old girl with persistent cyanosis. Data obtained by right heart catheterization reveal (a) transposition of great vessels, (b) intraventricular septal defect, and (c) patent ductus arteriosus. The problem of differential diagnosis in this case between the Eisenmenger complex and Taussig's heart, consisting of transposition of the aorta and overriding pulmonary artery, is outlined and the dynamics are discussed.

## 80. STUDIES ON THE SPREAD OF EXCITATION THROUGH THE VENTRICULAR MYOCARDIUM

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Direct lead electrocardiograms have been recorded on isolated perfused dogs' hearts subjected to certain injuries designed to influence the spread of the excitatory process through the ventricular myocardium. These injuries were produced by the introduction into the left or right ventricular cavity of solutions or crystals of potassium chloride, silver nitrate, phenol and cocaine.

Injuries sufficiently severe to be attended by the electrocardiographic pictures of bundle branch block did not evoke any specific effect which might be ascribed to arborization block consequent to destruction of Purkinje fibers. As complexes typical of bundle branch block developed in epicardial leads, the QRS complex in leads from the ventricular cavity into which the traumatizing agent had been introduced assumed an essentially monophasic character, with an R wave very similar to that recorded simultaneously from a contiguous epicardial lead. Apparently, endocardial and epicardial electrodes bore a similar spatial relationship to the spread of excitation through the intervening ventricular wall.

In an attempt to account for these findings, the following hypothesis was proposed. The speed at which the excitatory process spreads through a certain segment of the myocardial syncytium depends upon the orientation of the fibers in that segment to each other and to the point of origin of excitation. Spread down a strip of fibers of which the long axis is parallel to the long axis of the strip is rapid as compared with spread down a strip in which the fibers run at a right angle to the long axis of the strip. Endocardial activation is rapid not because of the presence of Purkinje fibers, but because the subendocardial bands of myocardium form a network through which excitation can move rapidly along the long axis of the fibers. Spread across the septum in bundle branch block and across the free wall of the left ventricle in the normally activated heart is slow because excitation is moving through fibers, the long axis of which is perpendicular to the advancing wave of excitation.

Testing of this hypothesis was undertaken by isolation of a segment of the ventricular wall, except for a limited attachment at one end of the strip. The spread of excitation down this segment was studied in relation to the disposition of the fibers constituting the strip. The presence of a band of fibers, the long axis of which is parallel to the long axis of the strip, appears to be essential to rapid excitation of the segment. Whether these fibers are epicardial or endocardial makes no difference.

## 81. THE PATHOLOGIC PHYSIOLOGY OF MEGA-ESOPHAGUS

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The actual underlying physiopathology of mega-esophagus is still a matter of uncertainty, in spite of many theories set forth. Because methods of determining the functional behavior of various parts of the esophagus have heretofore been so equivocal and inadequate, we have felt that esophageal motility studies by the balloon and kymograph method might aid in solving this problem. We have made extensive esophageal motility studies under varying conditions of three normal persons, three patients with achalasia, and five patients with organic obstructive lesions of the esophagus.

The three normal persons served as controls. They were studied under varying conditions over many hours. These studies show that organized frequent contractions of good amplitude are related normally with powerful peristaltic waves and an excellent deglutition effect, as revealed by a strong pull on the Einhorn tube attached to the balloon. They play an outstanding role in Magendie's third stage of swallowing.

Information obtained by fluoroscopy and x-ray film studies during barium swallow and by observed clinical effects on the inflated balloon which acted as a bolus of food was complementary to that obtained from the esophageal motility studies. The data considered together gave adequate conceptions of the action of the musculature of the esophagus. These data indicated that the intrinsic motility of mega-esophagus varies greatly, being almost always irregular, uncoordinated, hyporesponsive to stimuli, and not completely effective. In fact, the intrinsic motility at times completely failed, and in one patient was entirely absent, indicating total motor paralysis. This differs from that of the organic obstructive lesions of the esophagus in which there usually occurs a normal motility in the segment proximal to the lesion, but rarely a hypermotility or even a hypomotility, depending upon the degree of obstruction and the stage and complications of the organic disease. Achalasia of the esophagus also differs from true esophagospasm in which there usually occurs a hypermotility and hyperirritability.

These data indicate that the disease in idiopathic mega-esophagus is not localized necessarily to the cardiac end of the esophagus as is commonly believed. In our experience functional abnormalities often involve the entire length of the esophagus. These newer observations offer further evidence for, and are compatible with, the general theory of achalasia as an underlying mechanism of cause of idiopathic mega-esophagus.

A discussion will be presented of the effects of atropine and sympathicolytic and parasympathomimetic drugs upon the disturbed peristalsis. The clinical implications of these data will be set forth

## 82. THE PROTHROMBIN ACTIVITY OF HUMAN BLOOD

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In 1943, when the labile factor was discovered, it was noted that the addition of a small amount of deprothrombinized rabbit plasma, which has a high concentration of labile factor, to stored plasma reduced the prothrombin time markedly below normal. Recently it was found that if blood was carefully collected and stored in silicone-coated glassware, the prothrombin activity decreased on storage, but the addition of deprothrombinized rabbit plasma, that is, excess labile factor, reduced the prothrombin time only to the

level of fresh plasma. By means of the direct method of determining prothrombin by adsorption with tricalcium phosphate and elution with sodium citrate, it was further found that plasma stored in glass twenty-four hours or longer contained a much greater quantity of prothrombin than fresh or stored plasma kept in a silicone-coated container.

Obviously prothrombin activity increases in plasma stored in glass. This is masked by the decrease in labile factor and only becomes evident when labile factor is readded. In a silicone-coated container such an augmentation does not occur during several days of storage. The change in prothrombin activity is explained by postulating that prothrombin in circulating blood exists both in an active and in a precursor state. In contact with a rough surface such as glass the precursor is converted to the active form. In oxalated plasma the activation is relatively slow, while in native plasma it occurs in less than an hour. What factor other than the surface of the container is necessary for this conversion to active prothrombin remains undetermined. Platelets, calcium, and thrombin are not essential for this reaction.

Total and free prothrombin and the conversion to the active form are normal in hemophilia and thrombocytopenic purpura. In Dicumarol poisoning both active and total prothrombin are reduced. In one type of congenital hypoprothrombinemia both the active and total prothrombin are below normal; in a second type the active is low but the total may be normal.

In summary, prothrombin in human blood occurs partly in an active form and partly in a precursor state. Prothrombin activity as measured by the prothrombin time is dependent upon the concentrations of (1) active prothrombin (not total), (2) labile factor, and (3) bound calcium. Whether the inactive prothrombin acts as a reserve or becomes activated during standing is not known.

### 83. THE EOSINOPHIL RESPONSE IN ADRENOCORTICOTROPIC HORMONE (ACTH) THERAPY

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and John P. Rollins, M.D. (By Invitation), Chicago, Ill.

Ten patients with diagnoses of rheumatoid arthritis, ulcerative colitis, bronchial asthma, and other allergic disturbances were observed prior to, during, and, in some instances, following the cessation of therapy with ACTH-Armour by means of serial direct counting chamber eosinophil determinations of the peripheral blood with propylene glycol-phloxine stain diluting fluid as previously described by one of us (T. G. R.).

An average of eleven blood eosinophil determinations was made per patient prior to starting ACTH therapy. The average number of circulating eosinophils was found to be 472, the range varying from a minimum of 55 cells to a maximum of 2,354 cells per cubic millimeter of blood.

Eosinophils disappeared from the peripheral circulation following the intramuscular administration of ACTH in all instances, confirming the observations of Hills, Forsham, and Finch. An absence of circulating eosinophils was first noted in one case at six hours and in another at seventy-two hours, with an average of twenty-eight hours. The dosage of ACTH ranged from 40 to 200 mg. per day. The total quantity required for this eosinophil response varied from 15 mg. in one individual to 400 in another, averaging 112 mg. per twenty-four hours.

One patient was treated with 100 mg. daily for seventy-eight hours, two for fifty-four hours, receiving a total of 325 and 225 mg. ACTH; the eosinophils

returned to pretreatment levels three, four, and six days, respectively, following cessation of therapy. One patient treated at a level of 200 mg. daily for thirteen days developed an elevation in eosinophils to thrice his pretreatment average level coincident with a reduction of his dose to 150 mg.

Pretreatment examinations of the nasal secretions and sputa in three patients revealed high levels of eosinophils; these cells disappeared from the nasal and bronchial secretions between twelve and twenty-four hours after the onset of ACTH therapy

#### 84. ALLERGIC REACTIONS FOLLOWING THE INTRAVENOUS INJECTION OF CORN SUGAR (DEXTROSE OR GLUCOSE)

Theron G. Randolph, M.D., and John P. Rollins, M.D. (By Invitation), Chicago, Ill., and Clyde K. Walter, M.D. (By Invitation), Youngstown, Ohio

Pyrogen contamination has been considered as the major cause of reactions associated with intravenous dextrose therapy. Although the institution of sterile vacuum-packed fluid containers and sterile packed disposable recipient sets has largely controlled these factors, disturbing clinical reactions still occur. The fact that these reactions appear in selected individuals suggests that the cause may be due to some peculiarity of the recipient.

So completely have the terms dextrose and glucose been disassociated from their source material that it is not generally realized that *all* dextrose or glucose is prepared from the simple hydrolysis of corn starch. The fact that the ingestion of corn starch and corn sugar (dextrose or glucose) causes allergic responses in corn-sensitive individuals raises the possibility that corn sugar might also act as an allergen when it is injected.

Of several patients, previously shown to be highly corn sensitive, four were selected for this report. In each case the diagnosis of corn sensitivity was made as a result of the experimental feeding of corn meal gruel and corn sugar after four days of complete corn avoidance. Twenty-five cubic centimeters of 5 per cent dextrose were injected slowly intravenously under sterile and pyrogen-controlled conditions. In each case severe constitutional reactions occurred which were clinically similar to those observed following the ingestion of corn sugar and corn meal. The control injection of invert sugar of cane origin failed to produce a similar response with the exception of one patient clinically sensitive to both corn and cane. Similarly, the control injection of normal saline was without effect.

The importance of these observations cannot be dismissed inasmuch as corn sensitivity and wheat sensitivity are of approximately equal occurrence, ranking as the first two foods responsible for the production of chronic food allergy.

Clinical reactions from the intravenous injection of corn sugar occur both in undiagnosed and diagnosed cases of corn sensitivity. More violent reactions may be expected in the corn-sensitive individual who has avoided sources of corn in the diet, either intentionally or inadvertently, for several days prior to the intravenous administration of dextrose or glucose.

## 85. THE DETERMINATION OF THE BASAL METABOLISM BY PERIODIC MAXIMAL EXHALATIONS

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The measurement of the basal metabolism is subject to considerable variation through the natural inability of many subjects to breathe evenly. We have investigated the possibility that the chest position after a voluntarily forced exhalation might be less variable than after a quiet expiration and therefore lead to a more reliable method of estimating the basal oxygen consumption.

One hundred and fifty consecutive subjects of both sexes, with great diversity in age, height, weight, intelligence, and illness, were studied clinically. Their oxygen consumption was measured by the test and standard methods. The test method was a modification of the standard in that every minute the subject was directed to force a maximal exhalation. The first sixty-two subjects were tested by both methods. For the second group of fifty-four subjects a third method was introduced, which modified the test method by continuing for twelve or fourteen minutes and by including early in the test the performance of the maximal breathing capacity. The last group of thirty-four subjects performed the standard and test methods in duplicate. The order in which all tests were done in each series was determined from a prearranged schedule derived from a table of random numbers.

The test procedure does not measurably affect the rate of oxygen consumption. It results in significantly less variation between duplicates. It usually corrects for systematic or irregular deviations in the depth of undirected breathing that commonly lead to either an indeterminate conclusion or to a fallacious inference as to the true rate of oxygen consumption. The uniformity of the end point of forced exhalation is unaffected by such factors as lung disease, congestive heart failure, dysthyroidism, lack of training, or low intelligence of the subject and is less affected by consciousness of breathing than is the standard method.

Therefore this simple modification of the standard method of measuring the basal metabolism allows the rate of oxygen consumption to be estimated in some subjects when the standard method is unreliable, and in all subjects the estimate is usually significantly more precise.

## 86. IDIOPATHIC DILATATION OF THE PULMONARY ARTERY

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Increased interest in the differential diagnosis of congenital heart disease has attracted attention to an unusual disorder characterized by dilatation of the main pulmonary trunk with or without dilatation of the pulmonary arterial branches. This change in the pulmonary artery is unaccompanied by any other structural cardiac or pulmonary disease. It is considered a congenital cardiovascular disorder but its etiology is undetermined. Less than twenty cases which are acceptable by relatively strict criteria have been reported, but there is evidence to suggest that this anomaly is more common than has been assumed.

Five examples of idiopathic dilatation of the pulmonary artery have been observed. In two of them the diagnosis was established at autopsy and in

one of these the diagnosis was made ante mortem. This patient was observed over a period of ten years. The dilated pulmonary arteries were demonstrated angiographically six months before her death. At that time the right, main pulmonary arterial branch was completely occluded by a calcified thrombus. The post-mortem examination disclosed a pulmonary trunk which was actually considerably larger in diameter than the heart itself. The three additional patients are living, but the clinical picture seems compatible with the diagnosis in all respects.

This disorder seems to predominate in female subjects. It has been observed from childhood to old age. Dyspnea, cough, cyanosis, and hemoptysis are common symptoms, although the patient often has few complaints until late in his course, and the final illness may be very brief. The tolerance for exercise and pregnancy is usually surprisingly good. The major abnormalities disclosed by physical examination are a systolic murmur and a systolic pulsation in the pulmonic area, accentuation of the pulmonic second sound, and, often a diastolic murmur along the upper left sternal margin. The abnormally dilated right outflow tract is easily visualized radiographically as well as in angiocardiograms and abnormal pulsations of the pulmonary arteries are common. The electrocardiograms usually show incomplete or complete right bundle branch block or evidence of right ventricular hypertrophy. Recent studies by Cournand and associates have indicated that in these cases the systolic pressure within the dilated pulmonary artery is not elevated and, in fact, is lower than in the right ventricle.

#### 87. THE EFFECT OF AUREOMYCIN ON UROBILINOGEN FORMATION AND THE FECAL FLORA

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The effect of aureomycin on urobilinogen formation has been studied in a series of fifteen patients. The amount of fecal urobilinogen was determined serially in seven of these, single determinations being made in the remainder. Bacteriologic studies of the feces were carried out on eleven samples in four cases. In addition, the amount of urobilinogen in the bile obtained by duodenal tube was determined in eight cases.

These studies reveal that, following aureomycin, and concomitant with a great reduction or practical disappearance of coliform organisms in the feces, the urobilinogen of the feces, urine, and bile markedly diminishes, the values often falling into the range which characterizes complete biliary obstruction. The bile urobilinogen was entirely absent in seven of the eight instances, only a trace being demonstrated in the eighth. This may be contrasted with the uniform presence of small amounts in fifty individuals not receiving aureomycin. The Harrison test for bilirubin, applied to the feces, often became positive after aureomycin, but the amounts did not appear to correspond with the reduction in amount of urobilinogen.

In a case of subacute bacterial endocarditis in which urobilinogen studies were first carried out after one month of continuous aureomycin therapy, the feces contained very little bilirubin but gave a fairly intense Ehrlich reaction for urobilinogen. It was possible to demonstrate, however, that no sterobilinogen was present; polarimetry revealed that the only urobilin represented was dextrorotatory rather than levorotatory. It was believed significant that the bile was now Ehrlich negative, indicating that the fecal urobilinogen was not hepatic in origin. The feces at this time contained colon-aerogenes inter-

mediates but no typical *E. coli*. Nonproteolytic clostridia were also present. This combination is of interest since Kämmerer and Miller, and, more recently, Baumgärtel have ascribed the reduction of bilirubin in the colon to *E. coli* and spore-forming anaerobes. In the present study *E. coli* was regularly lacking in the feces after aureomycin and it is evidently essential to the reduction of bilirubin to stercobilinogen. It appears, however, that other organisms of aerogenes type are at times able, perhaps in association with clostridia, to effect a partial reduction of bilirubin, either to mesobilirubinogen or a dextrorotatory derivative thereof.

These results strongly support the belief that the urobilinogens of the feces, bile, and urine are wholly enterogenous. In recent years Baumgärtel and co-workers have described evidence suggesting that, of the two urobilinogens, mesobilirubinogen is hepatogenous, only stercobilinogen being enterogenous. The present study reveals, however, that aureomycin markedly depresses the total urobilinogen formation and that under its influence urobilinogen completely disappears from the bile. These findings are incompatible with the hepatogenous concept unless one were to assume that aureomycin interferes with liver function, for which there is no evidence.

## 88. PAROXYSMAL MYOHEMOGLOBINURIA WITH FATAL RENAL TUBULAR INJURY

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(INTRODUCED BY C. J. WATSON, M.D.)

A fatal case of paroxysmal myohemoglobinuria in a 23-year-old man is presented.

The history was suggestive of a familial occurrence. Fifteen cases have been described in the literature, in three of which there was a history of familial incidence.

Death was due to renal insufficiency and pulmonary edema. The myohemoglobinuria, which had been observed for the first three days of observation, disappeared six days before death. Myohemoglobinemia was carefully looked for but never demonstrated.

In the present case, the myohemoglobin in the urine was identified and distinguished from ordinary hemoglobin by virtue of its spectral distribution curve. Preliminary study of porphyrin metabolism revealed a moderate increase in fecal coproporphyrin, 60 per cent of which was type III.

Significant histologic changes were found in the striated muscles and kidneys. The uremia and subsequent death were the result of tubular injury, as indicated by the demonstration of hydropic degeneration of the proximal convoluted tubules. Although pigment casts were found, they did not appear to be sufficient in number to have been the cause of the uremia, as has been suggested by others. The finding of these casts supports the concept that the precipitation of pigment is more likely the result of disturbed renal function rather than the cause of it.

## 89. THE CLINICAL AND LABORATORY EFFECTS OF HYPOTONIC INTRAVENOUS INFUSIONS

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We have found hypotonic solutions given by vein effective in furnishing water whenever the customary amounts of sodium chloride and dextrose present in standard isotonic solutions were undesirable or unnecessary. The use of hypotonic solutions has, in our experience, been attended with good clinical results and has permitted a more exact maintenance of water and electrolyte balance. Hypotonic solutions of dextrose and/or sodium chloride were first used in correcting disturbances of the extracellular fluid (pattern disturbances, volume losses, and plain-water deficits) in diabetes. Further experience has shown that these solutions are equally valuable in the more seriously ill patients who have a large antecedent plain-water deficit or who have a continuing excessive demand for plain water. For the most part we employed solutions of one-half isotonic strength, and the three most commonly used solutions had the following composition: (1) 25 Gm. of dextrose per liter of distilled water; (2) 4.5 Gm. of sodium chloride per liter; (3) 12.5 Gm. of dextrose and 2.25 Gm. of sodium chloride per liter.

Thus far our clinical experience embraces more than fifty patients who have received hypotonic solutions in amounts varying from 1,000 to 6,000 c.c. in twenty-four hours over periods of from one to twenty-two days.

Immediate observations on the blood were made in twenty-three patients after 1,000 c.c. of a hypotonic solution had been given; twelve patients received 2.5 per cent dextrose and eleven received 0.45 per cent sodium chloride. The infusion was completed in periods of time varying from thirty to ninety minutes. Determinations of the plasma hemoglobin, total plasma protein, plasma specific gravity, hematocrit percentage, the number of erythrocytes, and the hemoglobin were made before the intravenous infusion was started, immediately after it was finished, and one hour after it was finished. As in earlier studies made after the prolonged use of large amounts of these solutions, but on blood drawn four to twelve hours after the last intravenous infusion had been given, none of these immediate determinations showed any evidence of harmful degrees of dilution. A transient, slight fall in their values, shown in most of the determinations made immediately at the end of the intravenous infusion, was followed in the majority by a tendency to rise toward the original levels at the end of one hour. The blood showed no plasma hemoglobin except in two patients; in the one in whom the experiment was repeated with the same intravenous solution, no plasma hemoglobin was found.

## 90. STELLATE BLOCK IN THE MANAGEMENT OF NARCOLEPSY AND CATAPLEXY

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CLEVELAND, OHIO

The effect on narcolepsy of the injection of procaine into the cervical sympathetic chains in four patients with narcolepsy and cataplexy, one patient with narcolepsy, and one patient with hypersomnolence forms the basis of this report. Because of observations on alterations of cerebral function by temporary interruption of sympathetic impulses to the brain, it was decided to ob-



serve the effect in narcolepsy. Associated symptoms and signs in these six subjects have been reviewed, and an attempt has been made to correlate these with the response to stellate blocks. Other common causes for excessive drowsiness were excluded by a number of laboratory procedures. An attempt was made to correlate the response of symptoms to stellate blocks and to the administration of Benzedrine and closely allied drugs.

The urinary excretion of gonadotrophic hormone (F.S.H.) prior to and following stellate blocks was studied whenever possible. An electroencephalogram tracing in one subject discloses a very abnormal deep sleep pattern prior to stellate block. Repetition of the electroencephalogram in this same subject after stellate block injection shows a distinct difference, with a tendency toward a more normal pattern.

In general, patients with narcolepsy and cataplexy have their symptoms controlled for a period of time following stellate block injection, but the effect is more favorable upon the narcolepsy than upon the cataplexy. No change in urinary gonadotrophin excretion was seen to follow the procedure.

#### 91. A SYNDROME OF HYPERTENSION, OBESITY, MENSTRUAL IRREGULARITIES, AND EVIDENCE OF ADRENAL CORTICAL HYPERFUNCTION

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AND HELEN E. CLARK, M.D. (BY INVITATION), ST. LOUIS, MO.

Twenty-four women suffering from arterial hypertension were found to possess certain common clinical and laboratory findings which suggested that they belonged to a previously undescribed symptom-complex. These were: (1) relatively sudden onset of obesity occurring at menarche, menopause, after multiple pregnancies or gynecologic operation; (2) obesity of the "central type," with pale striae on thighs and sometimes on upper arms; (3) menstrual irregularities; (4) good or excellent therapeutic response of blood pressure levels to diets low in salt, with reversals when the intake of salt was raised; (5) abnormally low concentrations of sodium and chloride in sweat. Secondary and less common signs and symptoms included an aversion to salty foods and high fluid intakes, unexplained periods of oliguria, inconstant glycosuria or a diabetic type of glucose tolerance curve which was accompanied usually by increased sensitivity of the blood sugar levels to intravenously injected insulin, plasma chloride levels above or at upper limits of normal, hirsutism, a tendency to easy bruising and ecchymoses, a relatively benign type of hypertension with little albuminuria or other signs of renal damage, and sensitivity of the blood pressure to injected desoxycorticosterone acetate or glucoside. In no instance were the cardinal manifestations of Cushing's syndrome present. Because these symptoms, signs, and findings were seen rarely in other types of hypertension and because concentrations of sodium chloride in sweat were normal in most subjects, including obese nonhypertensive women, it is believed that these patients represent a separate pathogenetic entity. The evidence is strongly suggestive that certain functions of the adrenal cortex are specifically disturbed in these patients, but not in other forms of hypertension or obesity.

## 92. A LONG-TERM EVALUATION OF THE THERAPY OF PERNICIOUS ANEMIA WITH FOLIC ACID

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AND BERTHE ARMSTRONG, M.D. (BY INVITATION), CHICAGO, ILL.

Folic acid (pteroylglutamic acid) was introduced in 1945 as a specific therapeutic agent for "liver extract principle" deficiency diseases. Numerous short-term observations quickly confirmed its value. Because the natural history of pernicious anemia is characterized by exacerbations and remissions, it was felt that long-term studies were necessary in order to critically evaluate any new therapeutic agent.

Early in 1946, ninety-eight patients with pernicious anemia were selected for such a study from the Out-patient Anemia Clinic of the Cook County Hospital. Their course, liver extract requirements, neurological status, and other characteristics were well established, the patients having been followed by the same group of observers and maintained in remission with liver extract for periods ranging from months to years. Folic acid was administered orally, in a daily dose of 5 milligrams. The patients were seen at four-week intervals for interviews and neurological and hematologic examinations.

The present report summarizes the observation made during the three and a half years the study has been in progress. There have been thirty-one neurological and thirty hematologic relapses, with eight patients showing a combination of both. In three patients the folic acid was discontinued because of persistent weight loss and/or soreness of the mouth. Twenty-nine patients discontinued their therapy for reasons beyond our control. Most of these patients disappeared from observation, many, however, only after insisting that they felt better while on parenteral liver therapy which they wished resumed. Of the ninety-eight patients only thirteen have been able to remain on continuous oral folic acid therapy and maintain themselves in remission.

Without exception, every hematologic relapse was quickly reversed with a change to parenteral liver therapy. In fourteen patients with neurological relapse, however, the changes have not been reversible in spite of intensive therapy with liver extract for six months or more. Bone marrow aspirations were performed in five patients at the time of relapse. Megaloblastic changes were demonstrable in all instances.

The original plan envisioned an attempt to increase the dose of folic acid in those patients who showed evidences of relapse, but the impression (gained from the current reports of the time) that larger doses of folic acid precipitated neurological relapses faster than small doses deterred us.

## 93. HEMOPOIETIC CHANGES DURING CHLOROMYCETIN ADMINISTRATION

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(BY INVITATION), LEE EHRLICH, M.D. (BY INVITATION), JAMES A. GONNER, M.D.  
(BY INVITATION), AND OSCAR FELSENFELD, M.D. (BY INVITATION)  
CHICAGO, ILL.

Toxic manifestations of oral administration of chloromycetin have not been reported. Recently we have observed three patients in whom hematologic abnormalities were encountered during chloromycetin therapy.

Two patients with typhoid fever and one with acute brucellosis were treated with oral chloromycetin for periods ranging from nine to nineteen days. The total dose of chloromycetin ranged from 30 to 60 Gm. and was given in a uniform dose of 3 Gm. per day (0.5 Gm. every four hours).

A significant fall in the white blood cell count, attributable to the diminution in the number of granulocytes, could be demonstrated as early as the sixth day of therapy. White cell counts as low as 3,000 and absolute neutrophil counts of 264 were observed. No significant changes in the total number of lymphocytes were demonstrated. A slight drop was seen in the red cell count and the hemoglobin. In one instance this drop was out of proportion to a fall ascribable to the underlying infection.

The marrow was studied in all patients. In the first, a marked granulopenia and left shift in erythropoiesis were present on the eleventh day of therapy. In the second patient the marrow was studied three and thirteen days after the discontinuance of the chloromycetin. On the third day a left shift in granulopoiesis as well as erythropoiesis was seen, with an increase in eosinophils. By the thirteenth day the left shift had disappeared in both series and evidences of marked regeneration accompanied by an extraordinary eosinophilia were seen. In the third patient, the marrow was studied at the beginning of therapy and revealed the changes expected with infection. Eleven days later (two days after discontinuance of therapy) granulopoiesis had markedly diminished and a left shift had occurred in erythropoiesis.

Upon the discontinuance of the chloromycetin there was an immediate spontaneous reversal of the downward trend in the blood values.

In the three patients studied there is evidence that within a few days chloromycetin produces a marrow hypoplasia more marked in the granulocyte series but also involving a maturation arrest of erythroid elements. The peripheral blood simply reflects the more fundamental changes in the marrow. In the therapeutic doses employed in the present series the marrow alterations are reversible.

Both clinical and experimental studies are in progress to verify and extend these findings.

#### 94. EXPERIMENTAL SYPHILIS IN THE RABBIT: THE RELATIONSHIP OF METACHROMASIA TO FIBRINOID DEGENERATION OF COLLAGEN AND THE LOCALIZATION OF SPIROCHETES IN THE TESTIS

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ST. LOUIS, MO.

Since the earliest studies of experimental syphilitic orchitis there has been recognized, as a characteristic feature of the inflammatory reaction in the testicular stroma, the deposition of a relatively acellular, loosely constructed material resembling Wharton's jelly. The term "mucous (or mucoid) degeneration" of the connective tissue has been applied to this change. The material stains metachromatically and has been found in greatest abundance in the tunica albuginea, particularly around blood vessels.

We have confirmed the preceding observations in a study of syphilitic orchitis in the rabbit using the Nichols' strain of *T. pallidum*. In addition, we have found that previous incubation of the sectioned tissue in hyaluronidase (Schering) abolishes the capacity for metachromatic staining with thionin and with toluidine blue. No metachromasia is found in the stroma of the nor-

mal testis. Focal fibrinoid degeneration of collagen has been observed in the metachromatic areas and the highest concentration of spirochetes has been noted in and around these sites.

It may be concluded that the loosely constructed, relatively acellular material which appears in large amounts in the stroma of the syphilitic rabbit testis and which stains metachromatically contains hyaluronic acid or a closely related substance. It would appear that the sites of localization of the metachromatically staining material, the fibrinoid degeneration of collagen, and the highest concentrations of spirochetes are intimately related spatially. A sequential study of syphilitic orchitis in the rabbit is now in progress to investigate the possible causal or temporal relationships between the multiplication of spirochetes, the development of metachromasia, and the appearance of fibrinoid degeneration of collagen.

### 95. CONTINUOUS DICUMAROL PROPHYLAXIS IN CORONARY DISEASE

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It seemed reasonable to suppose that an agent which would prolong coagulation time and have a deterrent effect on blood sludging might be of value in preventing thrombosis in chronic arterial disease. Accordingly, twenty-one patients have been given Dicumarol continuously for periods of three weeks to three years. Of these, eighteen had clinical and electrocardiographic evidence of coronary thrombosis with myocardial infarction, two had coronary sclerosis with angina of effort, and one had cerebral thrombosis with left hemiparesis.

After initial stabilization, prothrombin times were determined weekly by Quick's method, and Dicumarol was prescribed in daily doses sufficient to maintain prothrombin time between two and two and a half times normal control values. Individual requirement varied from 50 mg. every other day to 150 mg. daily.

Five patients showing marked sensitivity to Dicumarol and unable to take as much as 50 mg. daily without alarming fluctuation in prothrombin time were given menadione bisulfite, 2 mg. daily. These showed increase in tolerance, and prothrombin times thereafter were more readily controlled.

All patients were advised of the risks involved and instructed in the symptoms and signs of overdosage.

No disastrous toxic effects have been encountered. Transient hematuria occurred in three patients; rectal bleeding from hemorrhoids which had bled prior to therapy occurred in one patient; cutaneous ecchymoses occurred in three patients; epistaxis occurred in one patient. One man with previous history of bleeding duodenal ulcer bled following extraordinary dietary indiscretion although his prothrombin time was within the desired range. The drug was discontinued and coronary thrombosis recurred within ten days. Dicumarol was then resumed and he has been well and at work for the past ten months.

There have been no deaths in the series. Chest pain recurred in two patients when the drug was discontinued because of toxic effect and ceased when Dicumarol was resumed. Anginal attacks have ceased entirely in eight patients and have been markedly reduced in frequency and severity in two. All of the patients have resumed their former activities.

Electrocardiograms in five cases have shown a striking reversion toward normal.

It is surprising and worthy of note that all patients have followed instructions carefully and have expressed the desire to continue under treatment indefinitely in spite of the inconvenience involved.

Results seem to indicate that continuous and controlled Dicumarol therapy is practicable and may influence favorably the course of disease of the coronary arteries.

## 96. THE USE OF ORAL MERCUHYDRIN COMBINED WITH ASCORBIC ACID IN CARDIAC DECOMPENSATION

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The effect of the oral administration of a tablet containing 60 mg. of an inorganic mercurial compound (Mercuryhydrin), equivalent to 19.5 mg. of mercury, and combined with 100 mg. of ascorbic acid has been studied in one hundred patients with congestive heart failure. The patients' ages ranged from 12 to 60 years and the congestive failure was due to valvular, hypertensive, or arteriosclerotic heart disease. From one to three tablets were administered daily.

These tablets were equally effective as one containing 120 mg. of Mercuryhydrin alone. They were satisfactory in controlling mild congestive failure in approximately one-third of the patients when used as a primary treatment. They were effective in controlling mild to moderate congestive failure in approximately two-thirds of the patients after maximum compensation had been obtained following injectable Mercuryhydrin. They were usually ineffective in controlling severe failure when used alone, but frequently of value when used as a supplement to injections. Toxicity, when it occurred, was mild and transient in the large majority of instances.

The studies indicated that the tablets may be used either alone or as a supplement to parenteral therapy to assist in the control of congestive heart failure.

## 97. SMALL BOWEL CHANGES IN AMEBIASIS

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ERNA LEWIN-ARENDT, M.D. (BY INVITATION), CHICAGO, ILL.

Following an attack of amebiasis, many patients manifest a multiplicity of abdominal symptoms which, though nondescript and not too severe, frequently make them gastrointestinal problems. The frequent finding on cytologic examination of the stool of epithelial cells originating from the small bowel suggested the latter as the possible site of "lesions" leading to the mentioned symptoms. Hence, all patients with chronic amebiasis had x-ray films made of both the small and the large bowel for possible pathologic changes.

Of sixty patients with chronic amebiasis thus examined, twenty-seven showed changes from the normal in the small bowel, particularly the terminal ileum. These changes are not seen as a rule in ulcerative colitis, sprue, and various nutritional deficiencies.

Spasm and edema of the terminal ileum are the changes most often noted. More characteristic, however, are the edematous folds of the terminal ileum which sometimes made the terminal ileum appear almost as wide as the colon.

The nonspecific changes of segmentation and pooling as seen in sprue or the ulcerated involvement of the terminal ileum as seen in ulcerative colitis were not observed in these patients. The motility of the small bowel appeared undisturbed.

The patients demonstrating the described changes in the terminal ileum were in general more difficult to manage clinically, especially with regard to control of the cramping abdominal pain.

## 98. PHYSIOLOGIC AND PHARMACOLOGIC STUDIES IN A CASE OF PHEOCHROMOCYTOMA

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AND EUGENE B. FERRIS, M.D., CINCINNATI, OHIO

A case of pheochromocytoma, subsequently proved at operation and at autopsy, allowed us the opportunity to compare in a single patient the physiologic and pharmacologic specificity of a number of suggested diagnostic procedures. The patient, a woman with persistent hypertension for approximately one year, preceded by at least three years of paroxysmal hypertension, had a blood pressure which ranged from 215/140 to 250/155 mm. Hg.

Benzodioxane (933F) produced an immediate fall in pressure to normotensive levels. Dibenamine produced a similar fall, which was slower in onset and persisted longer. Tetraethylammonium chloride (TEAC) produced a sharp rise in pressure with failure to return to initial levels for some time; this rise was shown to be abolished by benzodioxane. Diminished sensitivity to the pressor activity of intravenous adrenalin was demonstrated. Histamine and mecholyl each caused a fall in pressure, the rise reported to be characteristic of pheochromocytoma being absent.

The results bear out the specificity of benzodioxane and Dibenamine in counteracting the pressor activity of circulating epinephrine. Response to benzodioxane seems more specific, however, because no depressor effects have been encountered in a series of control hypertensive subjects, whereas such reactions do occur with Dibenamine. Diminished sensitivity to exogenous adrenalin would also appear to have specific meaning in that tolerance to the effect of this drug appears to develop when it is present in the circulation in large amounts. The pressor response to TEAC would appear humoral in nature, specifically adrenergic in this instance since it was abolished by benzodioxane. The lack of a pressor response in this case to either histamine or mecholyl suggests that these tests are not specific for diagnosis.

The results suggest the specificity of benzodioxane in the diagnosis of pheochromocytoma, when the pressure is elevated, and the desirability of evaluating the mechanisms of pressor responses to histamine, TEAC, or other stimuli by administration of benzodioxane.

## 99. HEART BLOCK FOLLOWING MEDULLARY PERFUSION WITH BACTERIAL TOXINS

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The role of bacterial and viral agents in the production of heart block has been known for many years. Of particular interest has been the occurrence of bradycardia and heart block following acute bacterial infections, es-

pecially in the case of diphtheria. The question of whether myocardial damage per se or increased vagal tone or a combination of both is responsible has remained to conjecture, with the preponderance of opinion in support of cardiac damage. No adequately controlled experimental evidence has thus far been presented to note the effect of increased vagal tone in the pathogenesis of heart block.

Six commercially produced bacterial toxins were employed in this study, namely: *C. diphtheriae*, *Staph. aureus*, *Str. hemolyticus*, *Cl. tetani*, *Cl. welchii*, and *Cl. botulinum*. These toxins were perfused through the turtle brain which was connected with the body of the animal only by means of the vagus nerves.

Heartblock was produced with all of the toxins. The blocks produced by *C. diphtheriae*, *Cl. welchii*, and *Cl. botulinum* could be released by atropine. These results suggest an acetylcholine-like mechanism. The remaining toxins were not affected by atropine and the mechanism for their action is unknown.

#### 100. THE TAKATA-ARA REACTION IN THE DIFFERENTIAL DIAGNOSIS OF JAUNDICE

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In the work-up of jaundiced patients the Takata-Ara reaction (TAR) seemed of diagnostic help in certain types of jaundice; hence, an attempt was made to re-evaluate the differential diagnostic significance of the TAR on the basis of recent classification of hepatobiliary diseases.

The TAR was performed in 356 cases and compared with the results of cephalin-cholesterol flocculation, thymol turbidity, and zinc sulfate turbidity tests. The percentage of positive TAR's in the different types of hepatobiliary disease were as follows: Laënnec's nonfatty cirrhosis, 87.8; fatty cirrhosis, 35.5; postnecrotic (nonalcoholic) cirrhosis, 100; infectious viral hepatitis, 10.7; homologous serum hepatitis (HSH), 68.0; toxic hepatitis, 27.6; obstructive jaundice (incomplete, benign), 2.0; and obstructive jaundice (complete, malignant), 20.0.

The TAR may therefore assist not only in differentiating a cirrhosis with jaundice from an acute hepatitis but also from an infectious hepatitis. The TAR is not related to the degree of icterus, and the high incidence of abnormal TAR's in HSH is therefore not an expression of the well-known greater severity of the latter.

Moreover, no parallelism was found between the TAR and the cephalin-flocculation and thymol turbidity tests. A moderate parallelism existed between the TAR and the zinc sulfate turbidity, except in infectious hepatitis where the latter is moderately elevated and the TAR is negative; in toxic hepatitis this exception is less marked.

A discrepancy between the TAR and the zinc sulfate turbidity is also apparent during the recovery phase of hepatitis when the zinc sulfate turbidity increases while the TAR (if positive) becomes negative. While, in recovering hepatitis, a rise in the zinc sulfate turbidity and a fall in the cephalin-flocculation and thymol turbidity occurs, a rise in the TAR in addition (or the maintenance of a positive TAR) speaks for the transition from hepatitis to cirrhosis. Similarly, the appearance of positive TAR in a fatty cirrhosis seems to indicate progression to a Laënnec's nonfatty cirrhosis.

The TAR may indicate certain phenomena not demonstrable by the other flocculation tests.

101. A STUDY OF THE MOVEMENTS AND SOUNDS OF HEART VALVES OF VARIOUS LABORATORY ANIMALS (A MOTION PICTURE AND SOUND RECORDING)

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EDWARD J. BALDES, PH.D. (BY INVITATION), ROCHESTER, MINN.

The hearts of various laboratory animals were perfused with oxygenated Ringer-Locke solution and were kept beating for various lengths of time. Rather large openings were made in the walls of the auricles. This afforded an excellent view of the entire valves. Colored motion pictures were made of the exact movements of the mitral, tricuspid, aortic, and pulmonic valves. A sound record, an electric record of the sound, and an electrocardiogram were made at the same time the motion picture was made. These records enable one to see the movements of the heart valves, to hear the sounds they produce, to see an electric record of the sounds, and to see an electrocardiogram recorded at the same time. The results of this study furnish information that we believe will change some of our previous ideas about the factors that cause the mitral and tricuspid valves to open and close.

102. CARDIODYNAMIC AND RENAL CHANGES IN SPONTANEOUS AND NEPHROGENIC HYPERTENSIVE DOGS IN RESPONSE TO TISSUE INJURY

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Tissue injury, particularly abscess, produces a sustained fall in blood pressure in some spontaneous and nephrogenic hypertensive dogs. This study was undertaken to investigate the cardiodynamic and renal alterations ensuing during this depressor response.

The following determinations were done before and after various types of tissue injury: (1) renal plasma flow (R.P.F.), (2) glomerular filtration rate (G.F.R.), (3) plasma volume, (4) thiocyanate space, (5) central venous, right atrial, right ventricular, and pulmonary artery pressures, (6) blood pressure.

It is concluded from the data that: (1) Spontaneous and nephrogenic hypertensive dogs exhibit the same hemodynamic pattern. The cardiac output, plasma volume, thiocyanate space, and pulmonary artery pressure are all within normal ranges. Both types of hypertension are due to an increase in peripheral resistance. (2) Spontaneous hypertensive dogs all have normal G.F.R. and R.P.F. In contrast, some nephrogenic hypertensive dogs have normal clearances; others exhibit significantly reduced kidney function. (3) A sustained blood pressure fall can be readily elicited by injection of abscess-inducing substances such as turpentine, indicating that the depressor response is a nonspecific one with respect to presumed renal hypertensive mechanisms. (4) Accompanying the sustained depressor response in both nephrogenic and spontaneous hypertensive dogs is a sustained increase in R.P.F. G.F.R. does not change significantly. Renal vascular resistance, particularly efferent arteriolar, is reduced. (5) The plasma volume, thiocyanate space, venous pressure, and right heart pressures are not affected by tissue injury. The blood pressure fall is not due to hypovolemia. (6) The cardiac



output is increased during the depressor response to injury. The fall in blood pressure is therefore due to a decreased peripheral resistance. Apparently the decrease in both renal and general peripheral resistance is the resultant of a common train of events elicited by injury. There is no evidence for a cause and effect relationship between them. The blood pressure fall following injury cannot be attributed to "relief of renal ischemia."

### 103. CLINICAL EVALUATION OF A NEW LIPASE PREPARATION

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Many agents are available which have the property of digesting fat, yet there is a need for one which would be capable of hydrolyzing a wider variety of lipids.

It was thought, on the basis of experimental evidence in animals, that lipase A may be helpful in individuals who display an inability to adequately digest all forms of food fat. The new preparation is an enzyme, protein in nature, produced during the growth of a certain type of mold (fungus).

In this study lipase A was used in patients having steatorrhea. Control studies also were made. Each capsule contained 0.4 Gm. of the enzyme and each patient was given twenty-four capsules daily.

These individuals were placed on a calculated diet of low fat (50 Gm. in twenty-four hours) and evaluated clinically and chemically before and after lipase was given. The chemical determinations were made on the nitrogen, on the total and neutral fat and fatty acids of the stool; the blood was studied as to content of lipase, calcium, nonprotein nitrogen, and total protein, and the urine was studied as to total output of nitrogen. These determinations were made before and after the fat content was changed in the diet.

Comparative study was also made with the use of pancreatin in the same group of patients.

A specific example will demonstrate the efficacy of the new enzyme.

A patient with carcinoma of the pancreas was placed on a 55 Gm. fat diet plus adequate protein and carbohydrate. The total fat calculated in the stool before lipase A was administered was 57.0 per cent (dry method). After three capsules of lipase A were given per day for a period of three weeks, the total fat in the stool was reduced to 42.0 per cent. After the diet was increased to 152 Gm. fat, the stool fat was elevated to 73.0 per cent. The administration of twelve to twenty-four capsules of lipase decreased the total fat in the stool to 53 per cent and 40 per cent respectively.

Clinically, the patient improved, the stools decreased in number and became more solid, and the cramping in the abdomen subsided. In general, the patient ate better and felt better.

*Conclusion:* (1) Lipase A is helpful in steatorrhea. (2) Large quantities of the enzyme are required for optimum results. (3) Pancreatin compares favorably with results obtained with lipase A.

## 104. ANGIOCARDIOGRAPHY

GEORGE C. SUTTON, M.D., GEORGE WENDELL, M.D., HARRY GRANT, M.D., AND  
HAROLD WEDELL, M.D., CHICAGO, ILL.

(INTRODUCED BY DON C. SUTTON, M.D.)

A comparison is made between angiocardiology by arm vein and direct catheterization of the heart.

Direct intracardiac angiocardiology is a procedure possessing few difficulties and of negligible risk. The procedure will give contrast visualization of the heart and great vessels which is routinely superior to that obtained by peripheral injection of a radio-opaque material. This is especially true for levoangiocardigrams. Simple, inexpensive equipment for changing the films and low power x-ray apparatus are more practical for use with intracardiac injection, without loss of a very high percentage of diagnostic films. In some cases demanding a high concentration of contrast media and accurate timing of exposures, only intracardiac injection will produce satisfactory angiocardigrams.

105. DIVISION OF THE POPLITEAL VEIN IN VALVULAR  
INSUFFICIENCY OF THE DEEP VENOUS SYSTEM  
OF THE LOWER EXTREMITIES

GEZA DE TAKATS, M.D., AND GUSTAV W. GRAUPNER, M.D. (BY INVITATION)  
CHICAGO, ILL.

From a large number of patients exhibiting valvular incompetence of the deep veins, six patients have been selected to test the possibility that the division of the popliteal vein overcomes the "bursting" type of pain on standing and decreases or eliminates postural edema. All patients have had a deep thrombophlebitis with recanalization, subsequent edema, induration, and ulceration. All have been under our care for several years and were treated by compression bandages, excision of ulcers, skin grafts, or saphenous vein ligation, in spite of which there were recurrences. Since previous experience with femoral vein ligations gave no appreciable help, division at this site was done with the following results: (1) all six patients lost their "bursting" pain on standing, (2) swelling was greatly decreased or completely abolished in every instance, (3) patches of recurrent lymphedema with ulceration were uninfluenced, this complication requiring other forms of therapy. This simple procedure may eliminate the incapacitating sequelae of postphlebitic edema in selected cases.

106. QUANTITATIVE STUDIES OF TREATMENT OF ACUTE CLOSED  
CEREBRAL INJURY BY HYPERTONIC INTRAVENOUS  
GLUCOSE OR SURGICAL DECOMPRESSION

C. BRUCE TAYLOR, M.D., AND GEORGE M. HASS, M.D., CHICAGO, ILL.

Acute, closed, intracerebral lesions characterized by hemorrhage, necrosis, and progressive edema were produced in rabbits by freezing the brain through the intact skull with an instrument cooled by expanding carbon dioxide. The dimensions and locations of cerebral lesions were controlled so that they could be reproduced in successive animals.

19.5 per cent in 1943-44, and 18.6 in 1944-45. In 1945-46 and 1946-47 penicillin was used for treatment if there was no response to sulfonamide, or if sulfonamide drugs were contraindicated. Penicillin was used exclusively for treatment in 1947-48 and 1948-49. Mortality was 9.7 per cent in 1947-48, and in 1948-49, when aqueous crystalline penicillin in doses of 200,000 or 300,000 units was given intramuscularly twice on the first day of treatment and once daily thereafter, mortality was 5.7 per cent.

Pneumococcus bacteremia was found in 12.9 per cent of the cases in 1940-41, and in 49.5 per cent of the cases in 1947-48. In the remaining years bacteremia was found in 16.4 to 31.8 per cent of the cases.

Empyema, meningitis, endocarditis, lung abscess, or pyarthrosis occurred in 13.2 per cent of the cases in 1937-38, and in 3.8 per cent of the cases in 1940-41. In the remaining years these complications occurred in 4.6 to 10.7 per cent of the cases.

Apparently penicillin therapy has reduced mortality, and the unchanged frequency of bacteremia and purulent complications results from the onset of these manifestations before the start of treatment.

#### 109. CONTROL COMPARISON OF NU-2206 (3-HYDROXY-N-METHYLMORPHINAN HYDROBROMIDE) WITH MORPHINE SULFATE FOR RELIEF OF POSTOPERATIVE PAIN

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In an earlier investigation of the analgesic properties of NU-2206 (3-hydroxy-N-methylmorphinan hydrobromide) the effect of this agent on the pain threshold in normal volunteer student subjects was determined. There followed a clinical trial to determine its effect in a variety of patient categories. It was found to have desirable analgesic properties with an apparently more prolonged effect than that of morphine sulfate in equivalent dosage ranges. It was found that the dosage range was from 3 to 6 mg. as compared with 10 to 15 mg. of morphine. The side effects did not appear to be severe or frequent. A limited study was made on a small number of patients who had received the drug over a prolonged period of time, and no withdrawal symptoms were observed.

Its prolonged analgesic effect appeared beneficial in those patients who required protracted relief and in whom frequent injections with shorter-acting analgesics were undesirable. Controlled study then was undertaken on ward patients undergoing surgical procedures on the General Surgical and Urological services. Those under the age of five years or in such condition as to be outside the preselected dosage range were excluded. Patients having intracranial surgical procedures also were omitted. They were divided into fifteen operation categories so that subsequent statistical evaluation as to the amount of pain associated with various types of operative procedures could be compared. In each operation category alternate patients were given NU-2206 or morphine sulfate. The medications were so dispensed as to preclude identification except by code letter.

The nursing staff was instructed to give 1 c.c. for each patient or  $\frac{1}{2}$  c.c. for each patient. One cubic centimeter contained either 5 mg. of NU-2206 or 10 mg. of morphine sulfate. The drugs were administered by the nursing staff to be given as required for the relief of pain not more frequently than every three hours. A special form was provided listing seven common complications

ordinarily seen in conjunction with the use of analgesic drugs. The nurses' observations were checked closely by the same member of the staff each day.

It appears, after studying 406 patients by this method, that analgesic drugs are not needed in slightly more than one-third of the instances. A difference in duration of action of NU-2206 and morphine sulfate was not demonstrated. NU-2206 is as effective an analgesic agent as morphine, with no demonstrable increase in side actions.

## 110. SPONTANEOUS RUPTURE OF THE HEART FOLLOWING ACUTE MYOCARDIAL INFARCTION

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Cardiac rupture occurred in eight patients (7.2 per cent) of a series of 111 instances of acute myocardial infarction in patients who had been observed in the Evanston Hospital and came to autopsy during the past six years.

The eight patients were all of the white race. Their ages ranged from 51 to 86 years, averaging 68, and they were equally divided in sex. Half of the group denied prior angina, three had experienced pains from one to four months, and one who died within two days after onset of illness gave no history of anginal pain or hypertension and had considered herself in excellent health. However, there was evidence of old infarction in four of these patients.

Hypertension persisted after the onset of clinical myocardial infarction in all but one of these patients, and he belonged in the group of four who gave no prior history of hypertensive disease.

Death occurred rapidly after the occurrence of the rupture as evidenced by the condition of the pericardial sac and lacerated tissues. Four patients showed ante-mortem clotting of the blood in the pericardial sac and one had organization of the clot, but the remaining three revealed only bright, unclotted blood.

Clinical observation indicated that three expired immediately after the rupture, four within a day, and only one survived sixty hours. All eight deaths occurred within two to eleven days, average 5.8, after the clinical onset of acute myocardial infarction. The rupture occurred in the left ventricle in seven of the patients, four posteriorly and three anteriorly, while the remaining one ruptured through the right ventricle and septum at the apex.

Positive electrocardiographic evidence of acute myocardial infarction was present in all of the rupture group except one patient on whom such an examination was not possible because she expired within a half hour after admission to the hospital. The clinical diagnosis of rupture was made in six of the patients prior to autopsy.

Rupture of the heart occurred in four of these patients during defecation.

The leucocyte counts and blood sedimentation rates were consistently above normal levels in both the rupture and nonrupture groups, but there was no significant difference.

# 111. CLINICAL AND LABORATORY OBSERVATIONS IN FATTY INFILTRATION OF THE LIVER

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JEROME R. BERMAN, M.D. (BY INVITATION), DANIEL F. RICHFIELD, M.D.  
(BY INVITATION), FERDINAND G. WEISBROD, M.D. (BY INVITATION),  
AND EDWARD A. GALL, M.D. (BY INVITATION), CINCINNATI, OHIO

Fifteen patients with pure fatty infiltration of the liver demonstrated by needle biopsy were selected for study.

Thirteen of the fifteen patients were admitted to the medical service for various causes among which were hematemesis, pellagra, chronic cholangitis, diabetes mellitus, chronic pancreatitis, xanthomatosis, congestive heart failure, myocardial infarction, malnutrition, and gastritis. Two patients with mycosis fungoides and discoid lupus erythematosus respectively were studied on the dermatologic service.

The ages of the patients ranged from 29 to 85 years. Nine of the patients were women. A definite history of alcoholism was present in six. The diet was adequate in only six patients. An antecedent history of jaundice was obtained in two patients, both of whom had had previous cholecystectomies. Mild diabetes mellitus was present in two, while obesity was present in six patients. A history of weight loss of 10 pounds or more was obtained in ten of the subjects. Nausea and vomiting occurred in six, while anorexia was prominent in ten.

Hepatomegaly (ranging from one to five fingerbreadths) was observed in ten of the fifteen patients, while splenomegaly was present in only one instance. Edema of the lower extremities was encountered three times. Glossitis and evidences of peripheral neuritis were found in two patients.

Laboratory studies showed an elevation of the thymol turbidity in five of fourteen cases and a 3 to 4 plus cephalin cholesterol flocculation test in seven of fourteen cases. Serum bilirubin was elevated in two patients. Brom-sulfalein retention was demonstrated in seven of fourteen patients. The prothrombin time was not significantly prolonged in any instance. Anemia was present in six subjects and was macrocytic in two.

It may be concluded that fatty infiltration of the liver occurs in a variety of conditions and presents variable clinical and laboratory findings, and requires needle biopsy for its diagnosis.

# 112. CHANGES IN TOLERANCE FOR GLUCOSE AND IN THE MORPHOLOGY OF PANCREATIC ISLET CELLS INDUCED BY INTRAVENOUS GLUCOSE IN DOGS

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(INTRODUCED BY HENRY R. JACOBS, M.D.)

The possibility that damage to the islets of Langerhans can be caused by the continuance over long periods of time of glucose infusions in non-hyperglycemic amounts was the object of the present study and report. Previous investigators have shown that there is a response on the part of the blood sugar regulating mechanisms to administered glucose. It was postulated that continuous administration even in nonhyperglycemic amounts would elicit such a response in such a way that these mechanisms would have no chance to "rest" and would become exhausted.

Glucose was administered to female dogs by continuous intravenous infusion, and the blood sugar changes followed. The pancreas was examined microscopically at the end of each experiment. Short-term infusions with hyperglycemic rates of glucose input have shown that such continuous infusions of glucose will cause an increase in the tolerance for glucose, as evidenced by the markedly lower blood sugar seen at the end of such an infusion as compared with the blood sugar seen shortly after the infusion is started. Long-term infusions (as long as forty-eight days) of amounts well below the hyperglycemic glucose input were shown to cause pancreatic islet cell damage with beta cell degranulation, congestion, and hydropic degeneration, and resulted in a much poorer glucose tolerance as evidenced by much higher blood sugar levels, in the hyperglycemic range, at the end of a long-term infusion, than were caused by the same amount of glucose in the early part of a given infusion.

The results of this investigation would seem to indicate that the tolerance for glucose can indeed be increased, and that such an increased tolerance stimulated by continuous intravenous glucose infusion will result in damage to the islet cells of the pancreas, and eventual loss of glucose tolerance, probably due to exhaustion of the insulin producing cells in the islets of Langerhans. This same microscopic picture has been reported before as the result of hyperglycemia maintained over long periods of time, by both anterior pituitary extract injections, and again by injected glucose. It would seem from the results of this investigation that the damage and loss of tolerance for glucose is due not to the hyperglycemia but to the continued stimulation of the blood sugar regulating mechanisms.

### 113. THE TREATMENT OF UREMIA BY DIALYSIS ACROSS THE INTESTINAL MUCOSA

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(INTRODUCED BY N. C. GILBERT, M.D., AND GEORGE C. TURNBULL, M.D.)

The effects of dialysis across the intestinal mucosa by lavage are studied in two cases of chronic terminal uremia. The first is one of congenital polycystic kidney, studied for two months. The other is a case of advanced nephrosclerosis in a woman whose right kidney had been removed surgically years ago, studied for one month. The irrigating solution is lactate-Ringer's with added dextrose 5 to 10 per cent. Levels of nitrogenous products in the dialysate from small bowel irrigation are equal to blood levels and the abstraction rate is inversely proportional to the rate of flow. A large inflow (priming) is required in intestinal dialysis before outflow effectively equals inflow. Irrigation of the lower ileum and colon is equally efficient and allows for control of edema. Alteration of irrigating solution concentration of glucose controls edema during irrigation. Glucose is absorbed during dialysis, necessitating concentrations up to 10 per cent in the irrigating fluid to maintain isotonicity. Sodium chloride in 0.6 per cent concentration is absorbed at all levels of the intestine, but sodium chloride deficient solutions will abstract as much as 0.5 Gm. per liter. In view of this, an irrigating solution containing 0.45 per cent sodium chloride would seem more suitable. Sodium lactate in the dialyzing fluid in combating acidosis, hemoconcentration, or dilation during dialysis did not influence blood nitrogen levels. By fastening a small-bored tube four feet from the tip of a Miller-Abbott tube, inflow of dialyz-

ing fluid occurs through the former, and outflow through the latter, which can be allowed to remain at the ileocecal level. This was shown to cause no irritation although in place for one month. Blood nonprotein nitrogen rises rapidly in the uremic individual following intravenous administration of protein hydrolysate.

#### 114. A QUANTITATIVE STUDY OF THE SOLUBILITY OF HUMAN HEMOSIDERIN

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Previous studies of hemosiderin, isolated by drastic chemical methods, have indicated that the product is a form of ferric hydroxide. The present studies were an attempt to define the properties of hemosiderin by less drastic methods and to compare the properties of intracellular hemosiderin with those of chemically isolated hemosiderin and synthetic ferric hydroxide.

Hepatic tissue of patients with hemochromatosis and hemolytic hemosiderosis, secondary to multiple transfusions, was used. Frozen sections of fresh tissue were dried *in vacuo* for qualitative microscopic studies. Frozen pulverized fresh tissue was dried *in vacuo* for use in quantitative studies of solubility of hemosiderin. Sections for microscopic study and small amounts of powdered tissue were exposed to the action of buffer solutions of different ionic composition (pH 1.2 to 11; ionic strength, 0.25 and 0.50). Ferric hydroxide and hemosiderin isolated from hepatic tissue by the usual alkaline methods were exposed to similar solutions. Solubility was determined quantitatively by analyses of iron in supernatant solution and in the undissolved residue.

The data indicated that chemically isolated hemosiderin and ferric hydroxide had similar solubility properties. Hemosiderin in the cytoplasm of reticuloendothelial cells had solubility properties similar to but not identical with those of chemically isolated hemosiderin and ferric hydroxide. Hemosiderin in hepatic parenchymal cells had solubility properties which were very different from those of chemically isolated hemosiderin and ferric hydroxide. Intracellular hemosiderin, chemically isolated hemosiderin, and ferric hydroxide were almost insoluble in isotonic saline and phosphate buffer solutions at neutrality. A large fraction (50 to 80 per cent) of intracellular hemosiderin, especially that present in the cytoplasm of parenchymal cells, was soluble in citrate buffer solutions, ionic strength 0.50, at or near neutrality, while chemically isolated hemosiderin and ferric hydroxide were almost insoluble under the same conditions. Furthermore, within a part of the range in which intracellular hemosiderin had a high solubility (pH 6.1 to 7.0), crystals of unknown composition appeared in the tissues near the sites of granules of hemosiderin. These crystals did not appear in normal liver cells and were not formed in the presence of chemically isolated hemosiderin or ferric hydroxide. The results indicate that the problem of isolating one or more forms of hemosiderin by chemical extraction at neutrality, or after crystallization *in situ*, can now be approached.

## 115. INFARCTION OF THE MUSCLE BUNDLES OF THE HEART

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The object of this investigation was to determine whether myocardial infarcts are confined to the muscle bundles of the heart or occur without regard for them. For this purpose the topography of seventy-two infarcts in fifty unselected hearts was plotted and compared with the known topography of the four chief muscle bundles of the cardiac ventricles (superficial sinospiral, superficial bulbospiral, deep sinospiral, and deep bulbospiral muscles). It was found that all the infarcts followed definite patterns which coincided with the patterns of either one or more of the four muscle bundles. Twenty-five of the infarcts (35 per cent) in twenty-one cases (42 per cent) involved either a single superficial or a single deep bundle. The superficial muscles were involved, either alone or with a deep muscle, in 74 per cent of infarcts. The deep muscle bundles were involved, either alone or with a superficial muscle, in 51 per cent. Depending upon the thickness of the ventricular wall involved, it was possible to distinguish three types of infarcts: full thickness, massive but not full thickness, and laminar infarcts. Rupture, aneurysm formation, and mural thrombosis of the left ventricle were largely dependent upon the number of muscle bundles involved and the thickness of the infarct. Atrial infarcts were present in 42 per cent of the hearts. The immediate mortality of myocardial infarction was not influenced by the thickness of the infarct, the number of muscles included in it, nor by the involvement of any specific muscle.

116. INTERRELATION OF PTEROYLGLUTAMIC ACID AND VITAMIN B<sub>12</sub> IN INDUCED ANEMIA OF SWINE

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Swine maintained on a pteroylglutamic acid (PGA)-deficient diet, with "vitamin-free" casein as the source of protein, develop severe macrocytic anemia with megaloblasts in the bone marrow. Such animals respond well to PGA initially, but as the deficiency is maintained, the ability to respond to PGA diminishes. Administration of purified liver extracts then evokes a response. If liver extract or a source of "extrinsic factor" is given before PGA, a poor response can be elicited once or twice, after which no further response can be obtained until PGA is administered.

Two animals showed a slight response to relatively large doses of xanthopterin, but much less than to PGA.

One pig, maintained on the PGA-deficient regimen, for several months was given large amounts of liver extract and later vitamin B<sub>12</sub>. There was an initial partial response, after which blood values were maintained better than previously but never became normal. Very marked macrocytosis developed and persisted. Administration of PGA then caused prompt disappearance of macrocytosis, and blood values became normal.

Vitamin B<sub>12</sub> deficiency was induced in swine by a diet containing alpha protein of soy beans, supplemented with methionine, as the source of protein; PGA was administered daily. Severe anemia developed in which macrocytosis was less marked and developed more slowly than in the PGA-deficient animals. The marrow did not contain megaloblasts. These animals responded to crystal-



line vitamin B<sub>12</sub>. In one pig, there were peculiar double and triple reticulocyte peaks, each of which occurred after a single injection of vitamin B<sub>12</sub>.

These findings indicate that PGA-deficient swine fed "vitamin-free" casein develop a deficiency of both PGA and the antianemia factor(s) of liver (solely vitamin B<sub>12</sub>?). Either PGA or liver extract will induce a hematopoietic response, but both eventually are ineffectual unless the other is present. This indicates that both factors are required for normal hematopoiesis in swine.

While vitamin B<sub>12</sub> deficiency results in anemia, a deficiency of PGA appears to be importantly involved in macrocytosis and megaloblastosis. It is suggested that in human pernicious anemia a double deficiency also exists. The PGA deficiency may be the result of dietary inadequacy or of disordered metabolism of PGA. The deficiency of vitamin B<sub>12</sub> usually is dependent on an inadequacy of "intrinsic factor" in the gastric secretion but presumably can result from dietary deficiencies of vitamin B<sub>12</sub> and other factors.

#### 117. CORRELATION BETWEEN RIBOSE NUCLEIC ACID DEPLETION AND OTHER SIGNS OF LIVER DAMAGE AS INFLUENCED BY VITAMIN B<sub>12</sub>

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CHICAGO, ILL.

Disappearance of cytoplasmic ribonucleic acid compounds which are supposed to play a significant role in protein formation is one of the first signs of liver damage. Therefore, in comparison with twelve controls, the ribonucleic acid depletion (as recognized histologically) in thirty-six rats, forty-eight hours after intraperitoneal injection of a sublethal dose of carbon tetrachloride, was correlated with other signs of liver damage such as hydropic swelling of the parenchymal liver cells, increase of bromsulfalein retention, decrease of total liver proteins, and increase of histologically and chemically demonstrable fat. Chemical fractionation of total fat revealed the phospholipids remarkably constant, while all changes in the total fat were caused by variations of the neutral fat. Recent investigations had suggested that vitamin B<sub>12</sub> is important in the formation of ribonucleic acid compounds and subsequently it was demonstrated in this laboratory that the pathologic changes resulting from carbon tetrachloride (CCl<sub>4</sub>) intoxication was significantly less pronounced in rats which had previously received very large doses of vitamin B<sub>12</sub>. Therefore, forty-eight such protected rats were also included in the comparison.

In the individual animals of the intoxicated as well as the protected group, there was a parallelism in the degree of the different alterations. This parallelism was marked between the degree of fatty deposition and bromsulfalein retention. The increased bromsulfalein retention was found more related to circulatory embarrassment by the fat deposition (as judged from the presence of erythrocytes in the sinusoids) than to liver cell damage. On the other hand, there was an almost as marked parallelism between liver cell damage, ribonucleic acid depletion, and decrease in liver protein.

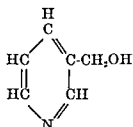
Since with small doses of CCl<sub>4</sub> ribonucleic acid depletion in the center of the lobules can be produced without other evidence of liver damage, the ribonucleic acid depletion is apparently one of the basic factors in this type of liver damage. Moreover, vitamin B<sub>12</sub> seems to inhibit liver damage by facilitating the formation of ribonucleic acid. That is probably related to the assumed role as synthesizing ribonucleases of growth factors such as vitamin B<sub>12</sub>. Their influence on protein formation in turn may control the enzyme regeneration in liver damage.

These experiences suggest the investigation of the effect of vitamin B<sub>12</sub> in different types of liver diseases. In view of the relatively large doses necessary for effect in rats, variations in vitamin B<sub>12</sub> content possibly explain the erratic results obtained with liver extracts in these disorders.

# 118. RONIACOL—A VASODILATOR SUBSTANCE CONVERTED IN THE ORGANISM TO NICOTINIC ACID

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Roniacol, a vasodilator substance which is converted in the organism to nicotinic acid, is 3 pyridine-methanol or B-pyridyl-carbinol (the alcohol corresponding to nicotinic acid) and has the following structural formula:



During the experimental period, the code designation for the compound was Nu 2121.

Roniacol is a nonvolatile solid freely soluble in water and in alcohol and of low toxicity for animals. Administration of the human dosage to dogs for three months produced no adverse effect on weight, blood formation, or nonprotein nitrogen. In all animals, powerful vasodilator effect on both coronary and peripheral circulation was observed.

There are several conditions in which prolonged vasodilation should be of benefit particularly in treatment but also in diagnosis. These include conditions in which coronary, renal, or peripheral cerebral vascular spasms are involved. Collateral circulation is of paramount importance when the vascular lumen is narrowed.

To be effective in aiding nutrition in an area with deficient vascular supply, brief and evanescent dilation in collateral vessels requires frequent repetition of the dilator effect. A prolonged dilator effect is to be sought and should aid in establishing permanent dilatation of the vessels involved.

Relatively free from uncomfortable or deleterious effects, and capable of causing an immediate, visible flushing of the skin when given by mouth, the drug can be repeated as often as desired.

This preliminary report is concerned with two conditions in which vasospasm and vasodilation in a collateral circulation are important, the angina pectoris syndrome and peripheral vascular lesions.

In angina pectoris, Roniacol has appeared repeatedly to increase tolerance for exercise and to extend the range of activity without occurrence of pain. In peripheral vascular disease the drug has been used to determine whether the total circulation of the involved parts can be improved; and, in cases showing improvement, sympathectomy may be expected to be effective.

Cases illustrative of the foregoing conditions are cited.

# 119. RAPID TREATMENT OF ACUTE GOUTY ARTHRITIS BY CONCURRENT ADMINISTRATION OF PITUITARY ADRENOCORTICOTROPIC HORMONE (ACTH) AND COLCHICINE

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RACHMIEL LEVINE, M.D., CHICAGO, ILL.

(INTRODUCED BY SAMUEL SOSKIN, M.D.)

Clinical gout appears to require two endocrine disturbances. An abnormal male sex hormone seems to regulate appearance of inherited hyperuricemia. Gout patients also appear relatively unable to meet acute relative glyco-corticoid lack with a prompt increase in production. When glyco-corticoid lack occurs, it tends to persist and to precipitate acute gouty arthritis. Hellman and Robinson, Conn, Block, and Louis induced acute glyco-corticoid lack by administering ACTH in interval gout and then withdrawing this hormone and found the procedure to precipitate acute gouty arthritis in a majority of patients. Their observations have been confirmed.

ACTH, given during acute gouty arthritis, repairs the glyco-corticoid lack and rapidly terminates the episode. However, given alone, ACTH is not satisfactory therapy for acute gouty arthritis. In most patients the relative glyco-corticoid deficiency which follows hormone withdrawal precipitates a renewed attack shortly after withdrawal.

Colchicine, given during or immediately after treatment of acute gouty arthritis with ACTH, effectively overcomes the tendency of ACTH withdrawal to precipitate a renewal of the attack. Single 50 mg. doses of ACTH (Armour) have effectively terminated most attacks within four hours. Occasionally a second or third dose has been required at six-hour intervals after the first. Although a number of the episodes treated had been prolonged and resistant to previous therapy, no ACTH-treated attack has persisted more than twenty-four hours after hormone therapy was begun.

Colchicine administration has been begun either with ACTH administration or four hours following the last dose of ACTH. All patients have been colchicized to tolerance and maintained on daily subtolerance doses of colchicine for at least two weeks. No patient who has received combined ACTH-colchicine therapy has had even a minor recurrence of acute gouty arthritis within one month of treatment.

Concurrent administration of ACTH and colchicine appears to be the most rapid and effective available treatment for acute gouty arthritis. In view of the relative scarcity of ACTH, the small amounts of this hormone which are required constitute a particularly desirable feature.

# 120. EFFECT OF HYPERVENTILATION ON THE HEMORESPIRATORY EXCHANGE IN NORMAL PERSONS, PATIENTS WITH PULMONARY EMPHYSEMA, AND PATIENTS WITH CARDIAC DYSPNEA

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(BY INVITATION), AND RICHARD V. EBERT, M.D.

MINNEAPOLIS, MINN.

Respiration aids in maintaining a normal blood pH by regulating the tension of carbon dioxide in the alveoli and hence in the arterial blood. Hyperventilation in normal persons produces alkalosis by sudden lowering of the alveolar and blood  $p\text{CO}_2$ .

The purpose of this study was to determine the response to voluntary hyperventilation of patients with dyspnea secondary to heart disease or chronic pulmonary emphysema. In twelve normal persons, fourteen cardiac patients, and twelve patients with pulmonary emphysema the total lung volume and its subdivisions, the minute volume, oxygen uptake,  $\text{CO}_2$  elimination, arterial blood pH,  $\text{CO}_2$  content, and oxygen saturation were measured in the basal state and after two minutes of voluntary hyperventilation. The  $\text{pCO}_2$  of arterial blood was estimated from the nomogram of Singer and Hastings.

In the group of patients with emphysema the residual air was markedly increased with a corresponding reduction in vital capacity, the total lung volume being normal. Patients with heart disease showed a reduction in vital capacity and total lung volume with a normal residual air.

Mean values before and after hyperventilation in normal persons and patients with cardiac dyspnea were very similar. In the group of patients with emphysema the initial  $\text{pCO}_2$  was elevated and the oxygen saturation was diminished, the value for the former being 50 mm. Hg and for the latter 83 per cent. The blood pH was normal as the result of a compensatory increase in plasma bicarbonate. Voluntary hyperventilation was relatively ineffective in this group and did not significantly alter the initial values.

Hence, patients with cardiac dyspnea like normal persons have ventilatory control over blood pH. Inability of patients with emphysema to increase alveolar ventilation effectively by maximal effort results in serious impairment of ventilatory regulation of blood pH. This defect renders these patients particularly vulnerable to metabolic and respiratory acidosis and is a constant threat to life.

## 121. A SINGLE SCALE ABSOLUTE READING EAR OXIMETER

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(INTRODUCED BY C. F. CODE, M.D.)

Two modified oximeter earpieces were calibrated on the basis of their responses on empirical optical filters using the single scale circuit described by Wood and Geraci.

Earpiece 1 was used to determine arterial oxygen saturation on thirty-three ears of eighteen normal subjects. Thirteen were white and five were Negroes. Ages ranged from 2 to 50 years. The average saturation obtained when the subjects were breathing air was  $96.7 \pm 0.3^*$  (93-101) per cent and  $99.4 \pm 0.4$  (94-106) per cent when breathing oxygen.

Earpiece 2 was used on twenty-four ears of seventeen normal subjects. Twelve were white, two were Chinese, and three were Negroes. The average arterial saturation was  $96.7 \pm 0.3$  (94-100) and  $99.7 \pm 0.5$  (96-104) per cent when the subjects were breathing air and oxygen, respectively.

Eighteen simultaneous Van Slyke and oximetric determinations of arterial oxygen saturation were made on six patients with congenital heart disease during supine rest, breathing air or oxygen, standing and walking. The per cent arterial oxygen saturation by Van Slyke analysis of radial artery blood averaged 85 and ranged from 39 to 100 per cent. The standard deviation of the differences between simultaneous Van Slyke and ear oximeter determinations in these hypoxemic patients was 4 per cent.

\*The number following the  $\pm$  sign is the standard error of the mean,  $N = 23$ .

The instrument can be A.C. or battery operated, is compact, relatively inexpensive, simple to operate, and is apparently accurate enough to warrant its use for most clinical estimations of arterial oxygen saturation.

## 122. STUDIES OF HEMAGGLUTININS IN CONGENITAL AND ACQUIRED HEMOLYTIC ICTERUS

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WITH THE TECHNICAL ASSISTANCE OF CHARLES A. CUNNINGHAM, B.S.

The immunohematologists studying the Rh mechanisms developed a series of techniques for demonstrating incomplete (blocking) antibodies which have proved useful in investigating other hemolytic anemias. To the Coomb's, "developing" and " " tests, Wheeler, Lubb, and Scholl have recently added a " " test previously suggested by Burnet, Pickels, and Quilligan, using enzyme-treated normal red blood cells for detecting incomplete Rh antibodies in sera. They found the technique a simple, reliable, sensitive, inexpensive, and rapid screening test for demonstrating incomplete Rh antibodies in sera of Rh-negative women. We have added this to the battery of techniques in the study of congenital and acquired hemolytic icterus. The following observations have been made: (1) 255 normal sera gave no agglutination with trypsinized red blood cells. (2) In twenty cases of acquired hemolytic icterus, one or both tests were positive in sixteen cases; fourteen with trypsinized red blood cells; fourteen with Coomb's serum. (3) In twenty-one cases of congenital hemolytic icterus, one or both tests were positive in eight cases; six with trypsinized red blood cells; four with Coomb's serum.

It is concluded that (1) the trypsinized red blood cell test is a valuable adjunct for demonstrating incomplete antibodies in acquired and congenital hemolytic icterus. (2) In acquired hemolytic icterus the avidity and titers of the trypsinized red blood cell test are generally greater than with the Coomb's test; however, they run a closer parallel in acquired than congenital types. (3) The Coomb's test, contrary to recent claims, has not been a reliable means of differentiating acquired and congenital hemolytic icterus thus far in our hands.

## 123. CAVITY POTENTIALS OF THE HUMAN VENTRICLES

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(INTRODUCED BY ROY W. SCOTT, M.D.)

The intracavitary potentials of the human left ventricle have been studied by retrograde arterial catheterization. In eight men, 48 to 74 years of age, the left ventricle was entered successfully in six, while in two the tip of the

\*The trypsinized red blood cell technique (modified after Wheeler): 1 ml. of washed (three times in cold normal saline) packed type "O" red blood cells is added to 1.5 ml. of a 0.1 per cent trypsin (Difco 1:250 activity trypsin) saline (buffered to pH 7.2) solution. Suspension incubated thirty minutes, 37° C., with frequent agitation. Centrifuge, remove supernatant, and wash enzymized red blood cells three times with cold saline. Make a 2 per cent cell suspension in saline. Suspension is checked for nonspecific agglutination with normal sera.

Serial dilutions of the patient's serum are made with 0.1 ml. of serum and 0.1 ml. of saline. Two drops of the above suspension of trypsinized red blood cells are added. Incubate at 37° C., thirty minutes, and read for agglutination.

Normal sera are run as controls.

catheter electrode was obstructed by the aortic valve. All patients had aortic insufficiency, with an average pulse pressure of 98 mm. Hg. The right ventricle potentials were obtained by venous catheterization.

In the region of the upper part of the interventricular septum, curves from the right ventricle showed a small R wave and a deep S wave; in the corresponding region of the left ventricle, the complex was of the QS variety, with an initial slurring on the downward limb corresponding to the R wave of the right ventricular lead. In no case was a positive deflection recorded in the left ventricle in this region. These findings are consistent with the current belief, proposed by Wilson, that the upper part of the interventricular septum is depolarized from left to right.

In one case, however, curves from the apex of the left ventricle showed a definite R wave and a deep S wave. The origin of this R wave is obscure and may be due to (1) depolarization of the lower septum from right to left, or (2) to the tip of the catheter electrode being situated in the apex which would be "facing" the wave of depolarization passing down the Purkinje system of the left ventricle.

Premature beats were most frequently produced when the catheter first entered the cavity of the left ventricle in the upper septal region. In several cases runs of ventricular beats occurred for two to four seconds, spontaneously disappearing when the catheter was withdrawn or passed forward toward the apex. Premature beats originating from the endocardium of the left ventricle produced a wide QS complex, and usually a positive QRS complex in the right and left arm unipolar leads and a negative QRS complex in the left foot unipolar lead.

Tracings were also obtained above the aortic ring and in the arch of the aorta. The ventricular complexes resembled those of the right arm unipolar lead. Thus in a patient with ST elevation in aVr, and depression of the ST segment in V<sub>3-6</sub>, and in aV<sub>1</sub>, the ST elevation in the lead from the arch of the aorta indicated that essentially cavity potentials were being recorded in aVr and aortic leads. However, aVr did not resemble the intracavitary lead in all cases. One patient with marked left axis deviation and left ventricular hypertrophy had positive aVr and aV<sub>1</sub>, but the cavity leads of the right and left ventricles had net negative values.

## 124. A STUDY OF PULMONARY HEMODYNAMICS DURING PNEUMONECTOMY

### A PRELIMINARY REPORT

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(INTRODUCED BY ROY W. SCOTT, M.D.)

Cournand has studied pulmonary hemodynamics before and after pneumonectomy in human beings, but there are no published data on the changes occurring during the actual operative procedure. We have studied five patients undergoing pneumonectomy by the technique of right heart catheterization during the operation, when a sudden burden may be expected to be thrown on the vascular bed of the remaining lung following ligation of the opposite vessel. A catheter is placed in the pulmonary artery opposite to the one to be ligated sixty minutes before the induction of anesthesia. Basal cardiac output and initial pressures are recorded and subsequent pressure

levels are determined at fifteen-minute intervals throughout the entire procedure, including the period immediately following ligation of the pulmonary artery. Intra-arterial brachial pressure, phase of respiration, pulmonary arterial pressure, and the electrocardiogram are recorded simultaneously on a six-channel oscillograph described elsewhere. In two patients who had lesions which were not resectable, pulmonary artery pressure measurements were obtained after temporary ligation of one pulmonary artery.

We have demonstrated a consistent average rise in pulmonary arterial pressure of 50 per cent above the basal level in all instances immediately following ligation. Pulmonary artery pressures dropped to near basal levels by the end of the operation in the three patients in whom resections were done. This took an average time of forty-five minutes. These findings agree with the recent work of Long and associates in dogs.

We believe that the preoperative measurement of pulmonary arterial pressure is an important adjunct in the evaluation of the patient's suitability for pneumonectomy. This immediate rise in pulmonary arterial pressure, with the sudden load thrown on the right ventricle, may be responsible for unexpected deaths which occur after ligation of a pulmonary artery.

Scott and Zimmerman have shown that elevated pulmonary arterial pressures due to a variety of causes may be lowered by aminophylline. The effect of this drug administered prior to ligation of the pulmonary artery is under investigation.

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